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HISTOLOGICAL CHANGES FOLLOWING OVARIECTOMY IN MICE

I dba HIGH TUMOR STRAIN*

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PLATES 1 TO 3

(Received for publication, April 16, 1941)

The formation of nodular hyperplasia of the suprarenal cortex and the development of mammary tumors in dba mice ovariectomized at birth have been reported by us in a short paper (1). Since then further data have been secured which enable us to follow the suprarenal changes from their earliest manifestation to the fully formed abnormalities and correlate these with the changes taking place in the vagina, uterus, and mammary glands.

The observations are based on data collected from 95 dba female mice which were ovariectomized at birth and killed at different ages. This particular strain has about 50 per cent breast tumor incidence in the virgin females.

Method

The ovariectomy was performed on the first post partum day. The mice were anesthetized by chilling. They were placed on a sheet of paper in the freezing compartment of an electric refrigerator for about 7 minutes. Small dorsal incisions were made and each ovary, together with its capsule and part of the oviduct, was removed. A dissecting microscope was used to aid in locating these organs. Each incision was closed by one stitch taken with fine white silk thread. The mice survived the operation well, and their mothers readily accepted and continued to take care of them.

At the autopsy of every animal a careful search was made to ascertain whether any ovarian regeneration had taken place. All suspicious areas were sectioned but in no case was ovarian regeneration found.

Changes in the Suprarenal Gland

In all the castrated animals killed after 3 to 4 months of age the suprarenals show characteristic changes which progressively lead to the formation of nodular hyperplasia. Most of the glands were studied on serial sections.

The earliest changes consist of the hypertrophy of a few cells of the zona

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glomerulosa Normally the cells of this zone are small and have relatively large compact nuclei and slightly basophilic cytoplasm Their cell outlines are indistinct After undergoing hypertrophy the nuclei become large and vesicular The cytoplasm increases in amount, accumulates lipid, stains lighter, and the cell outlines are more distinct Such cells occasionally are found in the process of cell division (Fig 1) Simultaneously groups of small, densely arranged cells appear immediately below the capsule and spread around the hypertrophied cells These subcapsular cells are polyhedral in shape, having deeply staining round nuclei and slightly basophilic cytoplasm They soon extend in between the cell columns of the zona fasciculata and here they become spindle-shaped and strikingly resemble the cells of the ovarian stroma As the subcapsular cells increase in number by mitosis they are seen first to interrupt the normal arrangement of the cells of the zona fasciculata, and later to invade and replace the cells of all three zones of the cortex This invasion soon results in the formation of a wedge-shaped abnormal area such as is shown in Fig 2 After the apex of the wedge reaches the medulla the sinusoid circulation of this part is disturbed and large blood-filled lakes are often found surrounding the abnormal areas The changes described frequently occur at 2 or 3 different parts of the cortex

Gradually the small polyhedral subcapsular cells of the abnormal area increase considerably in size, the cytoplasm accumulates lipid and the nucleus becomes vesicular (Fig 3) Later the enlarged cells form groups of cell nests, which are surrounded by spindle-shaped cells (Fig 4) At this stage these enlarged cells are indistinguishable from the hypertrophied zona glomerulosa cells By the increase in the size of the cell components the wedge-shaped areas change into rounded nodules, and often involve large parts of the cortex (Fig 5) The structure of such nodules resembles those lutein cells of mouse ovaries which develop from atretic follicles The cytoplasm of some of these cells often contains yellow pigment Occasionally the pigmented cells fuse and form large multinuclear giant cells

In some cases the enlarging abnormal nodules cause bulging of the surface (Fig 6) In other cases the nodules project toward the center of the gland and push the medulla into an eccentric position In several glands enlargements occur in both directions (Fig 7) Still further growth results in the invasion and finally in the breaking through of the capsule, after which a mushroom-like outgrowth occurs and results in the involvement of the surrounding adipose tissue (Fig 8) In extreme cases large areas of the surrounding tissues are involved Since the circulation is seriously disturbed, necrosis, thrombosis, and calcification in the central area have been seen to occur (Fig 9)

The same suprarenal frequently contains several abnormal nodules which may show different phases of the changes described Even in the most

extreme involvement some normal cortical and medullary tissue is always present. The advanced changes are visible in the gross, enlarging the gland and giving it a nodular outline.

Diffuse carcinoma of the suprarenal appeared in four animals. In three of these mice one gland was entirely involved, while the other gland showed nodular hyperplasia. In the carcinomatous glands two kinds of cells predominate: (1) The first type of cell is usually present at the periphery and at some places forms adenomatous areas. These are small polyhedral cells with dark staining oval nuclei, containing coarse chromatin granules, which are very similar to the subcapsular cells, present at an early stage of nodular hyperplasia. (2) The second type of cell forms the central part of the tumors and is arranged in diffuse rounded areas. In these cells the cell outlines are indistinct, the oval and vesicular nuclei are lighter staining and contain very fine chromatin granules. Both areas show mitotic activity. In one of these tumors areas of necrosis and calcium deposits were found. Metastasis was not observed. All three of these animals were between the ages of 18 and 20 months.

In a fourth animal belonging in the same age group and showing far advanced nodular hyperplasia, a small area of diffuse carcinoma composed of the first type of cells showing very active growth was present (Fig. 9).

Changes in the Uterus

The uterus remains undeveloped and has a very small diameter until about 6 to 7 months of age. Microscopic sections during this period show the typical slit like lumen, lined by inactive epithelial cells, the small atrophic uterine glands, and the dense cellular lamina propria which are so characteristic of this organ in castrated animals (Fig. 10).

The gradual changes which begin to take place between 7 and 12 months after castration are identical with those induced in the uterus of castrated animals by the injections of estrogen. An increase in the diameter is noticeable in the gross. Microscopic observations show an enlarged uterine lumen, an increase in the height of the epithelial cells and active cell division. The uterine glands also undergo similar changes. The lamina propria becomes greatly thickened by marked edema and hyperemia and, therefore, appears less cellular. The thickening of the muscle layers is mainly due to a size increase of the smooth muscle fibers, although occasionally mitosis also occurs (Fig. 11). The increase in size of the uterus reaches its height between about 11 and 16 months. Fig. 12 shows a section of the uterus at 15 months after castration. The epithelial cells show considerable mitotic activity and edema of the lamina propria is marked.

In many animals the uterine glands are greatly dilated and contain secretion. After about 550 days there is a slight decrease in size and hyperemia is less

marked Individual variations exist in the degree of development, which seem to be correlated with the abnormal condition of the suprarenal glands

Changes in the Vagina

Vaginal smears of several animals were examined Estrous cycles were detected, although they occurred irregularly Many of the vaginas were sectioned and examined microscopically The observed changes taking place here were also comparable with the changes which can be induced by estrogen injections in the adult ovariectomized animals

TABLE I

This table indicates the approximate time of the changes taking place in the suprarenal and the accessory sex organs The observations did not include all the accessory sex organs of all animals

Age	Suprarenal				Uterus			Vagina			Mammary glands examined microscopically			Tumors of mammary glands		
	No of mice examined	Normal		Nodular hyperplasia	No of mice examined	Typical castrate		No of mice examined	Typical castrate		No of mice examined	Under developed	Stimulated	Adenoma	Carcinoma	Other tumors
		per cent	per cent			per cent	per cent		per cent	per cent						
mos		per cent	per cent	per cent		per cent	per cent		per cent	per cent		per cent	per cent			
3-6	6	33 3	66 6		6	100		4	100		6	100				
6-9	5		40	60	3	66 6	33 3	3	66 6	33 3	4	75	25			2
9-12	6		16 4	83 6	16	16 4	83 6	3		100	5		100			3
12-15	14		14 2	85 8	13	30 7	69 3	1		100	7		100	2		11
15-18	23			100	19		100	8		100	15		100	1	9	4
18-21	30			100	25		100	19		100	25		100	1	18	16
21+	11			100	10	10	90	4		100	6		100	1	5	1

The epithelial lining of the vagina remains in an atrophic condition until about 6 to 7 months after castration Gradually marked thickening of the stratified epithelial lining is noticeable Cornification, mucification, as well as invasion with leukocytes, are easily recognizable phases

The Mammary Glands

All mammary glands of the experimental animals were examined by palpation and the glands of 68 mice were studied microscopically In general the glands remained rudimentary for about 8 months, and were comparable in size to the gland system of a 3 weeks old female After that period the ducts gradually lengthened and the formation of end-bulbs occurred In many

cases the ducts proximal to the nipples were irregularly dilated. Later, side-branches developed from the preexisting ducts, and some alveolar arcs were formed. An unevenness in the length and branching of the different glands of the same animal was frequently noticed. The youngest mouse to show a malignant mammary tumor was 14 months of age. Between the ages of 14 and 27 months the autopsy records of 75 animals show that 37 of them had tumors of the mammary glands (3 adenomas and 34 carcinomas).

Table I shows the data in tabulated form.

DISCUSSION

It is a generally accepted fact that the endocrine function of the ovaries dominates the uterus, vagina, and the mammary glands. Post pubertal ovariectomy results in the cessation of cyclic activities and in degeneration of these organs. The degree of atrophy is proportional to the time which passed between ovariectomy and autopsy (2). After injections of estrogen, rapid changes take place in the uterus and vagina and result in a recovery of these organs from the castrate condition (3). According to Allen (4) these changes are valuable criteria of ovarian endocrine function.

In the present experiment, although the animals were ovariectomized at birth and careful examination showed that no ovarian regeneration has taken place, the vagina, uterus, and mammary gland slowly recovered from the castrate condition and reached a state which they presumably can attain only under the influence of estrogen. The probability, therefore, exists that estrogen originated in some other organ in the absence of ovaries. The consistent nodular hyperplasia of the suprarenal cortex and close morphological similarity of these nodules to lutein like cells of the ovaries points to the abnormal suprarenals as the possible source of estrogen.

A comparison of our results with the experiments conducted by Parkes and Brambell (5, 6) is instructive. These investigators exposed the ovaries of mice at birth to x ray irradiation. The sterile ovaries evidently produced estrogen since the uteri and vaginas of these animals killed at adult ages did not show castration changes, but appeared stimulated. Estrous cycles developed in a normal manner and ceased after ovariectomy. In trying to analyze which ovarian elements were responsible for the production of estrogen the investigators came to the conclusion that the cords of cells which, after irradiation, proliferated from the germinal epithelium together with their connective tissue sheaths were the probable chief source. They state that the tissue of this "first proliferation often becomes like luteal tissue, the epithelial cells becoming like luteal cells, with connective elements of the sheath growing in amongst them like the thecal cells of the corpus luteum." The photomicrograph which illustrates this luteal like ovarian tissue in their article has a striking resemblance to the structure of the areas of suprarenal nodular

hyperplasia of our experimental animals. Fig 13 shows such an area for comparison. The close and common regional embryonic origin of the ovary and suprarenal cortex might account for the possibility that proliferating undifferentiated subcapsular cells of the suprarenal produced areas which were not only similar in structure to the ovaries but also in hormone production.

The time element in the production of these suprarenal areas is evidently an important one. The formation of hyperplastic nodules is a slow, gradual process, and the effect of estrogen on the uterus, vagina, and mammary glands can be definitely detected only after about 8 to 10 months. It is possible that late castration does not leave sufficient life span for markedly effective changes.

It seems important to emphasize the possibility that all strains of mice do not have the capability in the same degree for suprarenal changes following castration. Some evidence is already at hand pointing to the fact that in the C57 black mice, castrated at birth, the vagina, uterus, and mammary glands do not in all cases recover from the castrate state.

Clinical data seem to indicate that suprarenal cortical tumors in women lead to virilism. However, it should be kept in mind that to detect the effect of estrogen-producing components of abnormal suprarenals in the presence of even perhaps atrophied sterile ovaries would be more difficult. That such possibility might exist should be kept in mind. Frank (7) described two cases in which a large carcinoma of the suprarenal cortex in women was accompanied by increased amount of estrogen excretion in the urine. Pregnancy tests were negative.

In man a feminizing effect of suprarenal cortical tumors expressed by the enlargement of the breasts has been noted. Simpson (8) summarized five such reports and added a new case history. He stated that in his case "the adrenal tumor and its metastases were the source of the estrogenic hormone." In all these cases, where observations of the testes are included, it was stated that they were smaller than normal.

The feminizing effect of hyperplastic suprarenal nodules in castrated male mice has been observed and reported by us (9) and will be described more fully in a later paper.

CONCLUSIONS

- 1 In dba mice ovariectomized at birth the vagina, uterus, and mammary glands showed a gradual recovery from the castrate state, and finally reached the stage which they presumably can attain only under the influence of estrogenic hormones. Tumors of the mammary glands developed in 37 animals, of 75 examined, between the ages of 14 and 28 months (3 adenomas and 34 carcinomas).

- 2 As ovarian regeneration had not taken place the probability that estrogen originated in some other organ in the absence of the ovaries is suggested.

3 The consistent nodular hyperplasia of the suprarenal cortex and close morphological similarity of cells of these nodules to lutein like cells of the ovaries points to the abnormal suprarenals as possible sources of the estrogenic hormones

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EXPLANATION OF PLATES

All the sections used for illustration were stained with hematoxylin and eosin

PLATE 1

FIG 1 Suprarenal of a 3 months old mouse showing hypertrophy of a group of zona glomerulosa cells. Note the mitotic figure. $\times 600$

FIG 2 Suprarenal of a 7½ months old mouse showing the wedge-shaped area formed by proliferating subcapsular cells. $\times 300$



(Tékete *et al* Histological changes following ovariectomy I)

PLATE 2

FIG 3 Suprarenal of an 8 months old mouse showing beginning hypertrophy of the subcapsular cells in the wedge-shaped area $\times 300$

FIG 4 Suprarenal of a $10\frac{1}{2}$ months old mouse showing groups of enlarged cells surrounded by spindle shaped cells $\times 275$

FIG 5 Suprarenal of a $10\frac{1}{2}$ months old mouse showing a round nodule of hyperplasia extending from the capsule to the medulla and showing typical blood lakes $\times 100$



PLATE 3

FIG 6 Suprarenal of a 19 months old mouse with advanced nodular hyperplasia $\times 20$

FIG 7 Suprarenal of a 20 months old mouse showing the extension of abnormal nodules toward the center and toward the periphery of the gland. Note that the capsule has been broken through and the surrounding adipose tissue invaded $\times 20$

FIG 8 Suprarenal of a 12 months old mouse showing that the abnormal area broke through the capsule and invaded the surrounding adipose tissue $\times 66$

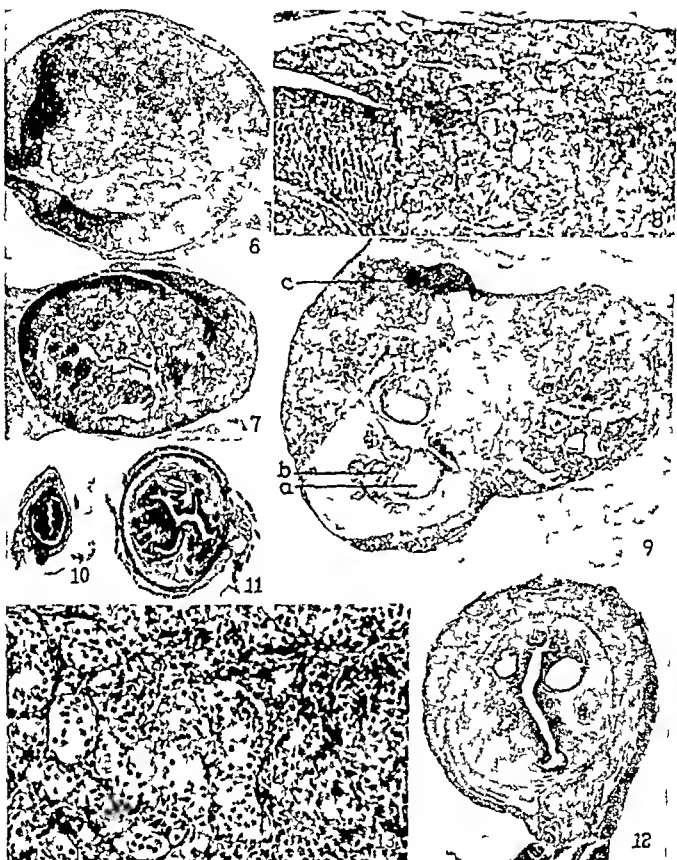
FIG 9 Suprarenal of a 20 months old mouse showing extreme case of nodular hyperplasia accompanied by thrombosis (*a*) and calcification (*b*). Note the small area of diffuse carcinoma at (*c*) $\times 20$

FIG 10 Cross section of the uterus of a 4 months old mouse showing typical castrate state $\times 20$

FIG 11 Cross section of the uterus of a 10½ months old mouse showing recovery from the castrate state $\times 20$

FIG 12 Cross section of the uterus of a 15 months old mouse showing enlarged uterine glands $\times 20$

FIG 13 Same suprarenal as shown on Fig 8, at higher magnification to show structural details $\times 183$



(Fekete *et al* Histological changes following ovariectomy 1)

Recently in this laboratory methods have been developed (8, 9) by which fluids in microscopic amounts can be brought into contact with the tissues of living skin at atmospheric pressure, or at various known pressures, in such a way that they enter neither the blood vessels nor the lymphatics directly. Under these conditions Locke's solution, at atmospheric pressure, is absorbed into the tissues intermittently (8, 9) and we have been able to measure the rate of its absorption. The technique has afforded an opportunity, as will be explained below, to determine with accuracy the resistance of the tissues to the interstitial movement of various fluids brought into contact with them. Observations have also been made upon the changes in the interstitial movement of Locke's solution and other fluids when brought into contact with the tissues under various positive pressures. The findings throw some light upon the nature of the interstitial spaces and the magnitude of the pressures operating in the tissues during the formation of lymph.

Methods

The technique by which exceedingly small amounts of test fluids have been brought into contact with the tissues has already been described (8, 9). It will suffice to recall here that a gauge 30 needle, carrying fluid from a horizontally placed 0.2 cc pipette, was introduced into the connective tissue of the skin in such a manner that the fluid brought into contact with the tissues did not pass into the blood vessels or the lymphatics directly (8). The movement of fluid in the pipette, occasioned by the entrance of the former into the tissues, was observed through a microscope and measured by the aid of micrometer eyepieces. As the amount of fluid entering the skin was exceedingly small, it was necessary to prevent all movement of the meniscus in the pipette resulting from expansion or contraction of the fluid following the slightest change in room temperature. Accordingly, the apparatus was submerged in a constant-temperature bath (8). An apparatus was also devised, as already described (8), by which the fluid in the pipette and hence the fluid brought into contact with the tissues could be subjected to various pressures. The detailed description of this device, already given (8), need not be repeated here.

The Effects of Pressure upon the Movement of Locke's Solution through the Tissues of the Skin

In 60 experiments we observed the effects of changes in pressure upon the movement of Locke's solution through the tissues of the skin of living mice. It is to be recalled that Locke's solution is readily absorbed from the tissues (9). The experiments yielded consistent results and need not be detailed individually.

The needle and pipette of the injecting device were filled with Locke's solution at atmospheric pressure and the needle carrying the fluid was introduced into the connective tissue of the skin of the ear or the back of mice anesthetized with nembutal (8, 9). The meniscus of the Locke's solution in the pipette was watched continu-

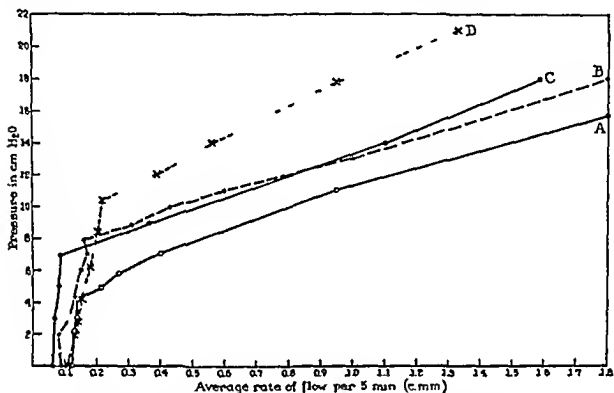
ously In all of the experiments it was noted, as in our previous work (9), that the Locke's solution at atmospheric pressure entered the tissues intermittently When the intermittent entrance of fluid into the skin had been observed for 15 to 20 minutes, that is, long enough to determine its rate and character, the pressure of a column of water 1.0 to 2.5 cm. in height was put upon the fluid in the injecting device, by means already described (8) The subsequent movement into the tissues was observed for 10 to 20 minutes, after which pressure in the pipette was again increased by small amounts for an equal period Later this procedure was repeated, employing slightly larger or smaller increments of pressure and observing the character and rate of fluid movement all the while until pressures of 20 to 40 cm. of water had been utilized The findings from two experiments typical of the sixty done are presented in Text figs. 1 a and 2 a, in which the data are plotted as described in our previous papers (8, 9)

In all the experiments the fluid continued to move into the skin at approximately the same rate as before when pressures of 1.0 to 2.5 cm. of water were applied, and in about half of them the intermittency of flow remained unchanged as well An instance of this sort is illustrated by Text fig. 1 a For the first 20 minutes of the experiment the Locke's solution, at atmospheric pressure, entered the tissues intermittently at quite regular intervals From the 20th to the 35th minutes, inclusive, as indicated by the numerals between the horizontal arrows near the top of the text figure, a pressure of 2.5 cm. of water was put upon the fluid in the injection apparatus Neither the rate nor the character of the flow changed appreciably

In the other half of the experiments pressures of 1.0 to 2.5 cm. of water produced slight alterations in the intermittency of flow, but the rate of flow did not increase The periods of inflow lasted a little longer, or recurred at shorter intervals, and the periods of no flow were correspondingly shortened The data from a typical experiment of this sort appear in Text fig. 2 a In certain instances, of which this is one, the changes just mentioned became more obvious when increasing pressure was put upon the Locke's solution (*Vide* Text fig. 2 a, from the 30th to the 45th minutes, when a pressure of 3.5 cm. of water was employed in the injection apparatus It will be seen that the rate of flow into the tissues increased only slightly)

In practically all of the experiments the flow became continuous when the pressure was raised to approximately 4.5 cm. of water Text figs. 1 a and 2 a both show this fact The intervals of no fluid movement were replaced by periods of steady flow, separated from one another by periods of slightly greater inflow It was as if a continuous passage through the tissues had been superimposed upon the ordinary intermittent inflow In about half the experiments, as *e.g.* in that charted in Text fig. 1 a, there was no change in the rate of entrance of Locke's solution into the tissues, while in the remainder, as typified in Text fig. 2 a, there was an insignificant increase In all of the experiments further increases of pressure up to 7.5 cm. of water produced hardly any further change, the intake of Locke's solution increasing but little and in many instances (none of which is shown in the text figures) not at all

Upon the application of low pressures, from 20 cm of water upward until the "breaking point" was reached, there occurred very little increase in the rate of flow, sometimes none. After the "breaking point" had been reached, each addition of pressure produced such an increase in flow that there was a linear relationship between the two, as though the latter were taking place through small channels. This interpretation will be discussed more fully below.



TEXT FIG. 3 The data from four typical experiments, plotted as in Text figs 1 b and 2 b, to illustrate further the effect of changes in pressure upon the rate of entrance of Locke's solution into the skin. Instances of high, low, and average "breaking point" (see text) have been selected. There was a linear relationship between pressure and flow after the "breaking point" had been exceeded.

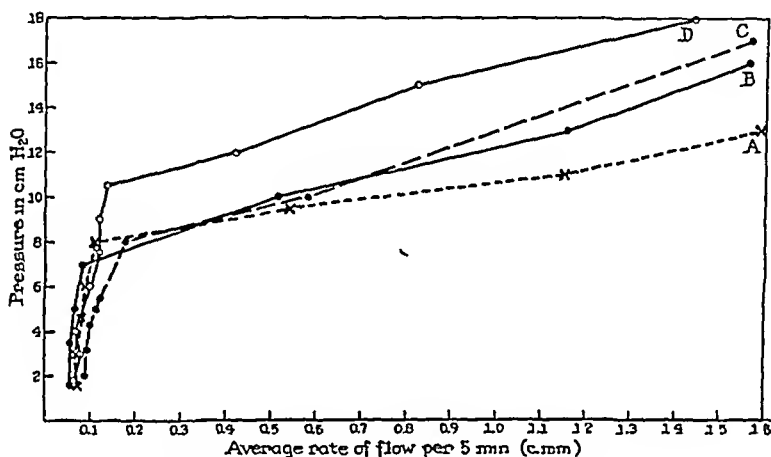
The Resistance of the Tissues to the Entrance of a Relatively Unabsorbable Edema Producing Fluid

In the work just reported, such minute amounts of fluid were introduced into the skin, even under pressure, that one could not be certain how much of the increased flow from the apparatus was due to a greater movement of fluid through the tissues and how much resulted from absorption by the blood vessels. The next experiments bore upon this point. They were carried out with a fluid similar in viscosity to Locke's solution but one which calls forth edema, augmenting the fluid bulk within the tissues.

To accomplish our end we have utilized a finding made previously in this laboratory and reported in an earlier paper (9). It was found that the addition to Locke's

have charted the data from this same experiment to show the average rate of inflow for each 5 minute period at the various pressures employed, up to 15 cm of water

Text-fig 5 shows the data from four typical experiments of the sort plotted in Text-fig 4 *b*. In some of the four, as e.g. in this experiment, there was little change in either the rate or the manner of fluid entrance into the skin at pressures below the "breaking point", in some the rate of flow increased slightly, while in others an increase in pressure resulted in a decrease in the observed flow of fluid into the skin. It is clear from this that the observed



TEXT-FIG 5 Changes in the average rate of entrance of dye-Locke's solution at various pressures. The data are plotted as in the preceding text-figure. Each increase in pressure after the "breaking point" had been exceeded brought about a corresponding increase of interstitial flow, as evidenced by the straightness of the lines.

differences in flow at different pressures below the "breaking point" often fell within the margin of error of the method. In all the experiments, after the "breaking point" had been reached each increase in pressure brought about a corresponding increase in the flow of the dye-Locke's solution through the tissues. As result, the later slant of the curves is approximately straight.

Resistance to the Entrance of Serum

Experiments like those just described were repeated, using homologous serum, a relatively unabsorbable and viscous fluid. It is well known that serum injected interstitially is absorbed slowly.

Fresh, sterile mouse serum obtained from pooled specimens of mouse blood taken with aseptic precautions was brought into contact with the tissues of the skin of the

ears or thighs of 34 anesthetized mice. In about half the number, as in previously reported work (9), the serum, at atmospheric pressure, entered the tissues at the extremely slow rate of 0.01 to 0.02 c.mm. per 5 minutes, about a third the rate of Locke's solution under similar conditions. In the other tests no fluid entered. Only these instances were employed in the present work. After 15 to 20 minutes had elapsed with no entrance of fluid, the pressure within the apparatus was raised by stages, as in the preceding experiments, until at last flow took place at a rate like that of the spontaneous flow of plain Locke's solution into the skin at atmospheric pressure. It was found that pressures of between 1.5 and 4.5 cm. of water sufficed to bring this about. The flow was continuous and slightly irregular like that occurring in the experiments made with dye Locke's solution as just described.

As in the experiments with the dye Locke's mixture or plain Locke's solution, further slight increases in pressure produced little increase in flow until a "breaking point" was arrived at. Then there occurred a sudden and greatly increased flow.

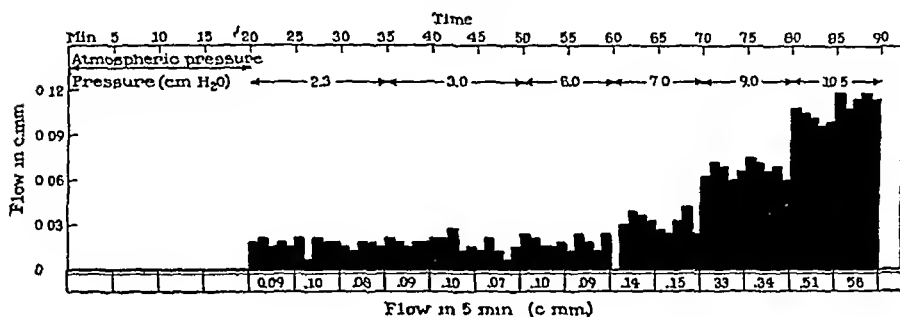
The findings of a typical experiment appear in Text figs. 6a and 6b. They show that the "breaking point," as indicated by the commencement of sudden, rapid inflow into the skin, occurred at approximately the same pressure as in the tests made with Locke's solution or with the dye Locke's mixture. In the experiments in which serum was employed the increase of inflow after the "breaking point" had been reached was not so great or so abrupt as in the trials employing the other solutions.

The Nature of the "Breaking Point"

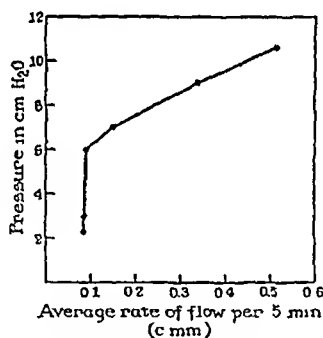
Findings in the Skin of Dead Animals—In the experiments so far described the "breaking point" was reached at the same pressure whatever the nature of the fluid employed. One may infer therefore that it was determined by the mechanics of the situation, by the bulk of the fluid introduced into the tissues whereby some structural change or separation of the formed elements was effectuated. To exclude the possibility that a circulatory change might have been responsible for the sudden entrance of fluid into the tissues, experiments like those described were repeated on animals that had been killed with ether 1 to 5 hours previously.

Plain Locke's solution, dye Locke's solution, and homologous serum were used respectively. We have already shown in a preceding paper (9) that Locke's solution and the dye-Locke's mixture at atmospheric pressure fail to enter the skin of killed mice. Homologous serum also has failed to enter in about half of the trials made, and it passed into the skin very slowly in the remainder. When pressure was brought to bear on these fluids, all three entered into the tissues continuously at pressures of 1.5 to 5.0 cm. of water and there was no sign of the intermittency of flow that appears when Locke's solution is brought into contact with living skin at atmospheric pressure or forced into it at low pressures.

Text-figs 7 and 8 (*a* and *b*) show the results in two typical experiments out of 26 made on killed mice. In the experiment illustrated by Text-fig 7, plain Locke's solution was employed, in that represented by Text-fig 8, a mixture of Locke's solution with $\frac{1}{2}$ per cent of dye. In both instances the test fluids failed to enter the tissues when brought into contact with the skin at atmospheric pressure. Subjected to pressures of 2.0 to 5.0 cm. of water, the fluids entered the tissues in a continuous



TEXT-FIG 6a

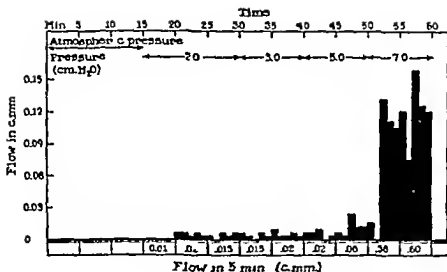


TEXT-FIG 6b

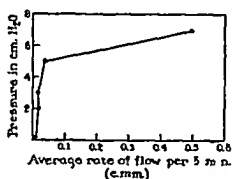
TEXT-FIGS 6a and b The rate of entrance of a relatively unabsorbable fluid homologous serum, at various pressures

manner, and there was almost no sign of the intermittent flow which takes place when Locke's solution is forced by similar pressures into living skin (9). It will be noted that in both experiments a sudden increase in the rate of entrance of the test fluids ("breaking point") appeared when the pressure of the introduced fluid was raised to 7.0 and 9.5 cm. of water, respectively.

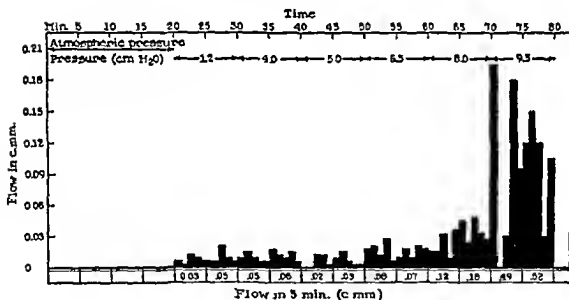
The findings in these two experiments were like those obtained with living animals except for the fact that plain Locke's solution did not enter the skin at atmospheric pressure. The other twenty-four experiments of this group yielded similar results. "Breaking points" appeared at the same pressures



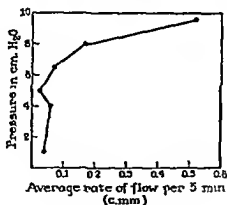
TEXT FIG 7a



TEXT FIG 7b



TEXT FIG 8a



TEXT FIG 8b

TEXT FIGS 7a, b, and 8a, b The rate of entrance of Locke's solution (Text fig 7) and of edema forming dye-Locke's solution (Text fig 8) at various pressures into the skin of recently killed mice

as in the experiments on living animals, that is to say, at a pressure of about 85 cm of water on the average. In rare instances "breaking points" appeared at a pressure of 50 cm of water or failed to appear until the pressure was raised to 120 cm of water. The findings showed clearly that the resistance to the entrance of fluid into the skin of recently killed animals is like that offered by the skin of the living. It follows that the circulation has nothing to do with it. The conclusion seems justified that the "breaking point" is due to the giving way of some structural barrier to inflow.

The Entrance of Fluid into Edematous Skin

Edema of the skin much affects the entrance of fluid

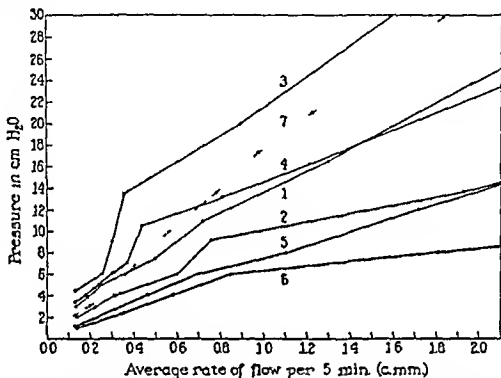
In 39 experiments edema was induced in the skin of the ear by painting it with xylol. This procedure had been found effective in inducing edema in scores of earlier experiments (10-14), as shown by the appearance of the skin under the microscope and by "pitting on pressure" exerted with a small needle. The edema was pronounced in the present experiments and the appearance of the skin proved a sufficient indicator.

In all of the 39 experiments the dye-Locke's solution was employed and observations on the entrance of fluid into the tissues were made as already described. In fourteen cases observations were begun 15 to 20 minutes after painting the ear with xylol, that is to say, while the edema was developing (12). In ten of the remaining 25 experiments tests were made $1\frac{1}{2}$ to 6 hours after painting the ear with xylol, in eight instances 20 to 24 hours later, and in seven instances 4 to 11 days later. In them all the test fluid was brought into contact with the connective tissue of the skin in the usual way and the fluid reservoir was then opened to the atmosphere, to find whether the tissue contained freely movable edema fluid under pressure. When that was the case it flowed back into the pipette. The backflow was always intermittent. It occurred in twelve of fourteen instances in which studies were made within an hour after painting with xylol, that is to say, while edema was developing. It also took place in all of the ten instances investigated $1\frac{1}{2}$ and 6 hours after induction of edema, but only in half of the eight studied 20 to 24 hours after the xylol painting, despite the fact that the ears were still swollen. Still later, 4 to 11 days after edema had been induced, backflow occurred into the injection apparatus in six of the seven trials made. In all seven edema of the skin was still visible at this time.

After it had been ascertained that edema fluid was present, the pressure in the injection apparatus was raised by small increments for varying periods of time and the changes in the rate of flow inwards of the dye-Locke's solution were followed as in the preceding experiments.

In Text-fig. 9 the findings from seven typical experiments are given, to show the changes in the rate of inflow of the dye-Locke's mixture when introduced into the edematous skin under various pressures. The curves are plotted as in Text-figs. 3 and 5. We have omitted from them the initial backflow into the apparatus which occurred in most of the experiments when the pipette was opened to the atmosphere. It is noteworthy that in most of the instances

there was no well defined elbow in the curve, at best an ill defined one, or often indeed none at all, like that which is indicative of the existence of a "breaking point" when fluid is forced into normal skin (*vide* Text figs 3 and 5 and the other figures plotted in the same way) The instances showing evidence of an elbow were usually those with the least or most recent edema, as will appear further on



TEXT FIG 9 The rate of entrance of dye Locke's solution at various pressures into edematous skin. The relationship of pressure to flow is plotted as in the preceding text figures of the same sort. In contrast with what they show, each small increase in pressure in the present instance was attended by a significant increase in the rate of flow. In some instances there was nevertheless a "breaking point" beyond which fluid entered faster.

Lines 1, 2, 3, and 4 represent the findings in four of the fourteen experiments made an hour or less after painting the ears with xylol, that is to say, during the formation of edema. Lines 1 and 2 represent typical findings from instances which showed much free edema fluid, as judged by the amount of backflow into the apparatus during the preliminary test of conditions. Line 3 is a typical curve plotted from the findings in an instance which showed very little edema fluid, and line 4 is taken from one of the two instances of the four teen studied during the development of edema, in which there was no backflow into the apparatus although the skin was obviously edematous. It is to be noted that lines 1 and 2 do not mount like lines 3 and 4, that is to say, relatively low pressures had a much greater effect on the entrance of fluid in the experiments from which the first pair of lines was drawn. Further, it is of

interest that lines plotted from experiments in which there was little or no demonstrable edema fluid, lines 3 and 4 respectively, show an elbow, as if the edema had not wholly done away with a "breaking point." These findings are typical of the data from experiments that have been omitted from the figure for the sake of simplicity. In about half of the instances showing much edema fluid the findings were generally like those plotted in line 1, yielding no evidence of a "breaking point," whereas in the others a fairly well defined elbow appeared, as in line 2, in spite of the presence of edema fluid.

The two lowest lines, 5 and 6, in Text-fig 9 show the findings in two instances typical of the ten experiments in which the tests were begun $1\frac{1}{2}$ to 6 hours after painting the ear with xylol. In every instance back-flow occurred at atmospheric pressure, showing that there was free fluid present in the skin. Lines 5 and 6 are drawn from experiments made 5 and 4 hours, respectively, after painting the ear with xylol. In both of these instances there was much back-flow into the apparatus at the beginning of the experiment when the dye-Locke's solution was brought into contact with the tissues at atmospheric pressure. The dotted line 7 gives the findings in an experiment made 5 hours after painting the ear with xylol. In this instance there was only a little demonstrable edema fluid and the line slants more sharply upward than lines 5 and 6. It is plain that the test fluid introduced into the skin in this experiment at increasing pressures did not pass into the tissues as readily as in the instances that showed much free edema fluid. Nevertheless there was no evidence of a "breaking point."

The findings from the experiments made 20 to 24 hours or more after painting the ears with xylol are not shown, for when plotted the data yielded lines similar to those numbered 5 and 6.

The findings plotted in Text-fig 9 have been selected as typifying the changes that occurred, but deviations were frequent. The rate of movement of fluid introduced at a given pressure was not always greatest when the skin contained most fluid as evidenced by the grade of edema, nor was the flow inwards at a given pressure always greater in ears edematous several hours than in those painted only an hour before. Some of the individual differences can be explained no doubt by differences in the pressure under which the edema fluid was held, or by differences in the interstitial pressure, a factor to be discussed in a later paper.

As already noted, in some instances if the edema fluid in the tissues was apparently scant, there was suggestive evidence of a partial "breaking point," as indicated by a suddenly lessened slant of the plotted lines.

On comparing Text-fig 9 with Text-figs 1 b to 8 b, inclusive, it will be seen that the lines in the latter run almost vertically in the early part of the experiments, showing that pressures between 20 and 80 cm. of water effected no significant increase in the movement of fluid into the skin. Not until the

"breaking point" was reached did a significant increase in flow take place. In Text fig 9, on the other hand, the slope of the lines shows that each small increase in pressure above that required to initiate flow into the tissues resulted in a significant increase in the rate of flow. This was true, to a greater or less extent, in all of the 39 experiments on edematous ears. The lines are far from vertical in the first portion of Text fig 9 and as the pressure was raised many of them became approximately straight. The significance of this difference will be discussed below.

DISCUSSION

The findings throw light upon the manner of movement of interstitial fluid through connective tissues. The resistance of dermal tissue to the entrance of the test fluids at the rate at which Locke's solution is taken up at atmospheric pressure was negligible. But the skin offered a definite obstacle to the entrance of fluid at a faster rate. Regardless of the fluid employed, the rate of flow into the skin did not increase appreciably as pressure was increased, until a "breaking point" was reached. Since no relationship was found between the rate of fluid entrance into the skin and the pressure employed until this point had been attained, the findings yielded nothing to suggest the presence of preformed spaces or channels through which fluid might stream. On the contrary they indicated that there are no such spaces or channels. But after the "breaking point" had been reached each further increase in pressure led to proportionate increases in the rate of flow of the introduced fluid irrespective of the character of this, with result that a linear relationship developed between the increases in pressure and the flow. This was roughly constant for each animal but differed from individual to individual, as the slope of the lines in the text figures show.

At pressures above the "breaking point" fluid moved through the tissues as though in small spaces or channels. From the fact that the "breaking point" occurred at the same level regardless of the fluid employed, one can infer that it was determined by the mechanics of the situation, by the bulk of the fluid introduced. The possibility that circulatory changes could account for it has been ruled out by the experiments which showed that it occurred at the same pressure in the skin of living animals and in those killed with ether. Obviously it signified a separation of the formed elements, resulting from the pressure of the introduced fluid. Inevitably such a change must always occur when fluids are injected into tissues by band, as in clinical medicine, for under these circumstances, as will be shown in later work, the pressure of injection is far higher than that required to exceed the "breaking point."

This abrupt change in the characteristics of fluid movement through the interstitial tissue of the skin makes plain a fact suggested by earlier work (13, 14), that interstitial fluid does not exist normally in tissue spaces large enough to permit it to move freely.

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A METHOD TO DETERMINE THE PERIPHERAL ARTERIAL BLOOD PRESSURE IN THE MOUSE

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PLATES 4 AND 5

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A method for the determination of the blood pressure of the mouse without injury to the animal will be briefly described in the present paper, together with certain observations upon the changes of pressure under various physiological conditions. The method has the advantage that it enables the pressure to be taken while injections into a vein of the tail are in progress.

Previous Work—Recently Bonsmann (1) reported a method to determine the blood pressure in the tails of mice and rats. A specially designed cuff enclosed the tail except for its tip which lay under a photoelectric cell. Blood was driven out of the tail by pressure in the cuff and when it was allowed to return the resulting change of color in the tip of the tail was registered by the cell and the pressure was read off. More will be said of Bonsmann's findings below. As the method employed the animal's tail, it was not suited to our purposes. Still more recently Diaz and Levy (2) and Williams, Harrison and Grollman (3) have published excellent methods to determine the blood pressure in the rat, but their methods also involve utilization of the animal's tail.

Griffith (4) has reported a method to measure the blood pressure in the legs of the rat. In his work the blood vessels of the skin of the foot were observed while pressure was exerted on the thigh by means of a cuff. We have modified the method of Griffith, observing the return of blood to the claws of the hind feet of mice.

The Method

The claws of the hind legs of mice were found to be transparent to the focused and cooled beam of a carbon arc light. Under these circumstances, as will be shown below, the entire circulation of the nail bed, including the afferent and efferent vessels and the capillaries, becomes visible under a binocular microscope. One can see with ease the moment at which peripheral circulation ceases in the claw, if pressure is exerted higher up on the leg by a cuff, as in clinical methods for determining blood pressure. One can note too with great accuracy the moment that arterial circulation begins anew when pressure is gradually released. Readings can be made more accurately by

observing the circulation in the claw than by watching the circulation in the skin of the leg, for, as Griffith (4) has pointed out for the rat, there is difficulty in determining whether or not the smaller cutaneous vessels seen under the microscope are arterial or venous¹

Mice of 16 to 35 gm body weight were anesthetized by a single intraperitoneal injection of nembutal or luminal. Nembutal was given as a 1 per cent solution, 0.5 cc per 25 gm of body weight, luminal as a 2 per cent solution in doses of 0.125 cc for every 10 gm of body weight. In the dosages employed, the luminal usually produced a deeper and longer anesthesia, but, as will be seen below, the depth of anesthesia varied greatly from animal to animal at any given time after the injection.

The Pressure Apparatus—It is difficult to arrange a blood pressure cuff about the upper part of the leg of small mice, for the leg is so shaped that the cuff when inflated tends to slip toward the foot. It was necessary to prevent this in some way. The following means was employed—

The board on which the anesthetized mouse lay consisted of pieces of cork cemented together. It was hollowed out, as shown in Fig. 1, and two movable strips of celluloid were laid over the hollow. These strips could be shifted to accommodate animals of different sizes. Two smaller celluloid plates, 6 cm long and 2 cm in height, were set upright and parallel to each other into other smaller pieces of cork board, as shown in the figure. These celluloid strips stood 1.5 cm apart. Through each plate of celluloid three holes were bored, 1 cm in diameter, large enough to allow the anesthetized animal's hind legs and tail to pass through without meeting obstruction. When the animal was placed on its back, as shown in the photograph, and one leg was drawn through the holes, a segment of the thigh 1.5 cm in length lay between the two celluloid plates and about 0.7 cm above that portion of cork board shown in the figure at A.

A small rubber blood pressure cuff had been constructed, of such size that when deflated it could be passed about the animal's thigh, just filling the space between the parallel pieces of celluloid and the board at A. A ribbon-like strip of steel, just wide enough to fit between the celluloid plates and bent in a semicircle, was placed over the cuff while the latter was still deflated, as indicated at C in Fig. 1. Through holes bored in both celluloid plates pins, not shown in the figure, were passed horizontally just above the metal strip. When inflation of the cuff was begun, the pins prevented the metal strip from rising. As result, the cuff was held against the leg by the metal strip above, by the cork board below, and by the celluloid plates at each side, and was so retained by these structures that pressure came upon that portion of the leg which lay between the celluloid plates. The latter, in addition, prevented the cuff from slipping up or down the leg. The cuff was connected with a mercury or water manometer in the usual way and with an inflating bulb.

To make visible the circulation in the nail, the rays of a Leitz carbon arc light were cooled by passage through 5 cm of water and focused, by an adjustable concave mirror, upon the claw to be examined. For simplicity this part of the apparatus has been omitted from the photograph in Fig. 1. The brilliant light transilluminated the

¹ It may be noted in passing that the circulation in the claw of rats, even of young ones, can be seen only with difficulty.

claw and when the room was in darkness the circulation of blood and movement of the individual red cells in the capillaries could be easily seen with the aid of a microscope. In each experiment the light was focused in turn upon each of the five claws and the circulation in each examined. The claw yielding the clearest picture of its blood vessels was selected. The blood vessels were best seen when the rays of light entered on the convex surface of the claw.

The vascularization of two claws is shown in Figs 2 and 3. To obtain Fig 2 an anesthetized mouse, 27 gm in body weight, was slowly injected into a vein with 1 cc of a solution of Higgins India ink diluted six times with a 0.9 per cent NaCl solution containing 1.3 per cent of gelatin. After this had entered the circulation, the animal was bled from the jugular vein while a second cubic centimeter of the ink solution was injected. A few minutes after the injection the foot was removed and placed in ice water for 15 minutes and then in cold alcohol for half an hour, after which it was partially cleared in methyl salicylate for 24 hours. The vessels, magnified 100 times as seen in the photograph, have approximately the same caliber as in life and appear much as they do during the measurement of blood pressure. In order to avoid dilatation of the vessels it was necessary to make an incomplete injection, and as result the picture does not bring out all the vessels present. Further, in a photograph it is impossible to include all the vessels since they cannot be brought into one plane. The richness of the circulatory bed of the claw is better demonstrated in Fig 3, which was obtained under the same circumstances as Fig 2, except for the fact that the injection material consisted of 4 cc of a mixture of 8 per cent gelatin solution with equal parts of undiluted Higgins India ink. It will be seen on comparison with Fig 2 that the smaller vessels are much dilated. During life blood flows toward the periphery in the smaller vessels of the plexus at the center of the picture and returns in the two large marginal vessels.

The measurement of blood pressure was carried out in the following manner. The cuff was rapidly inflated to a pressure estimated to be just above the systolic blood pressure, which will be seen below to vary somewhat with the stage of anesthesia and the condition of the animal. If the movement of blood continued in the claw, the pressure in the cuff was raised slowly until it ceased. When cessation of circulation was attained the pressure in the cuff was lowered by stages of 10 mm of mercury, allowing it to remain at each level for 2 minutes. When flow first appeared in any of the vessels of the claw, the pressure was recorded and the cuff deflated. After 2 or 3 minutes the cuff was again blown up, this time to a pressure a few millimeters of mercury higher than that just recorded. When all flow in the claw ceased, the pressure was lowered 2 or 3 mm. of mercury at a time, with a wait of a minute or two at each new pressure until a slight movement of red cells appeared in one or two of the small vessels in the central portion of the plexus near the base of the claw, the region indicated in Fig 3 by an arrow. The slight movement usually lasted for only

a few seconds and then ceased, as though it had been caused by a *readjustment* of fluid in the vessels and not by true blood flow. In most instances no further flow took place and a minute or two later the pressure in the cuff was lowered 2 or 3 mm. of mercury. As result, a pulsating flow of cells, toward the extremity of the toe, usually made its appearance, first in one or two channels, then in most of the vessels of the plexus. In some instances, without any lowering of the pressure, the slight initial movement of cells was followed by the pulsating flow, while in rare cases this failed to appear until the pressure had been lowered several times by 2 or 3 mm. of mercury. As a rule, a few seconds after the pulsating flow of cells began the flow became continuous through all the small channels of the plexus. For a little while, as the pressure was maintained in the cuff, the cells accumulated in the larger collecting vessels at the edge of the plexus, but after half a minute, or slightly more, blood flow usually established itself in these vessels too. If the circulation failed to become complete within one or two minutes the pressure in the cuff was reduced by 2 or 3 mm. of mercury and invariably flow appeared in all the vessels. If the final reduction of pressure was not made, the circulation eventually established itself, but sometimes only after several minutes.

For reasons to be discussed below, we have taken as the systolic blood pressure in the leg that pressure found in the cuff at the moment when the pulsating flow of blood first appeared in the small vessels of the claw and became continuous. As routine, to avoid undue congestion of the foot, pressure in the cuff was released as soon as the systolic pressure had been determined. In all instances two pressure determinations were regularly made in the manner just described and the average of the readings was taken, if agreement was good. When the readings varied by more than 5 mm. of mercury, a third determination was made and the three readings averaged.

Control Experiments

The question arose, were we measuring the true systolic pressure in the large arteries of the thigh or did the apparatus merely obstruct blood flow in the skin and claw? To test this point two series of control experiments were made. In the first, dye was injected into a tail vein while various pressures were maintained in the pressure cuff. In the second series of control experiments blood pressure was determined directly from the carotid artery and compared with simultaneous measurements obtained from the leg by the method just described.

Results of the Injection of Dye into the Circulation during Measurement of the Blood Pressure—In twelve experiments, after the systolic blood pressure had been measured in the leg as just described, the cuff was inflated to a pressure higher by 2 to 4 mm. of mercury. After ascertaining that flow in the claw had ceased, 0.05 cc. of a 5.4 per cent, isotonic solution of a vital dye, pontamine sky blue, was injected into the tail vein. This dye solution, the preparation of which has been described (5), has been used by us in larger or smaller doses in much previous work (5-8). The injections

employed here colored the animals well except in the portion of the leg and foot below the pressure cuff. After a few minutes, during which color still failed to appear in the occluded foot, the pressure in the sphygmomanometer cuff was lowered by a few millimeters of mercury until the pulsating flow of red cells made its appearance in the minute vessels of the claw.

This procedure invariably was attended by blue coloration of the leg and foot. This had been absent previously, showing that there was no blood flow to the tissues of the foot or lower leg at pressures above the one we accepted as the peripheral systolic pressure.

The second series of control experiments involved simultaneous blood pressure readings in the leg by the method described above, and in the carotid artery by direct cannulation of the latter.

Direct Measurement of the Carotid Blood Pressure of the Mouse—Mice anesthetized with nembutal or luminal were injected intravenously with 0.1 cc. of a heparin solution, 10 units to the cc. They were placed on the board shown in Fig. 1, covered with light layers of cotton, and kept warm by means of electric lights placed near the body. Half an hour later one carotid artery was exposed and cannulated with a gauge No. 27 hypodermic needle, employing a binocular microscope for its insertion. During this manipulation the artery was occluded with a rubber-tipped bulldog clamp. To prevent all loss of blood during the measurement of blood pressure, the apparatus shown in Fig. 1 was employed. The cannulating needle, previously filled with heparin solution, was connected with a three-way stopcock and this in turn was affixed to a 0.2 cc. Bureau of Standards pipette bent at right angles, as shown in the figure. The pipette was also filled with heparin solution but contained a minute droplet of mercury in the center of the graduated portion. A manometer in circuit with a device by which any desired pressure could be brought upon the contents of the pipette was connected with the latter as shown in the figure. The device need not be described in detail here as that has already been done in earlier work (9), while furthermore, the principles of its operation are clear from the photograph (Fig. 1).

During the insertion of the needle into the artery and while it was being tied in with very fine silk thread the stopcock was turned in such a way that the contents of the needle was shut off from that of the pipette. The clamp on the artery was then released and a pressure of about 100 mm. of mercury was put upon the contents of the pipette. The stopcock was then turned for a second or two to allow communication between the needle and the pipette-manometer system. Since the pressures in the pipette and the carotid artery were usually not equal, the droplet of mercury in the pipette began to move either toward or away from the artery. The stopcock was immediately closed and the pressure in the pipette-manometer circuit either increased or decreased by raising or lowering the leveling bulb *B*, shown in Fig. 1. Again the stopcock was opened to permit communication between the needle and the pipette and the droplet of mercury allowed to return to its original position. These adjustments were repeated as often as necessary until the pressure in the pipette just balanced that in the carotid artery and the mercury droplet merely pulsated in the pipette but did not flow in either direction.

A Comparison of the Findings Obtained by the Two Methods Just Described — In fourteen experiments simultaneous readings of blood pressure in the carotid artery and in the leg were successfully obtained. As a rule, the reading of the manometer attached to the carotid artery was a few millimeters higher than that attached to the cuff when the cells in the minute vessels of the claw first manifested the pulsating movement already described, which later, and without change in cuff pressure, became continuous. Better agreement between the two methods was sometimes found if the pressure in the cuff was read at the moment when the first irregular movement of cells occurred in the smallest vessels of the obstructed claw. But, as already mentioned, the first irregular movement of cells in the small vessels of the claw sometimes ceased and was not resumed again unless the cuff pressure was lowered by a few millimeters of mercury. It was felt that this movement might simply be the expression of readjustment of blood in the vessels following the early release of pressure in the cuff and the phenomenon was not as clear-cut as the appearance of the pulsating flow.

In some experiments many readings were taken by observation of the claw and while doing so the cuff was repeatedly inflated or deflated. In these instances pronounced edema of the foot and lower leg developed. Under these circumstances the first appearance in the capillaries of the pulsating flow which later became continuous occurred at cuff pressures which became progressively lower than the direct carotid blood pressure readings, eventually by as much as 10 to 15 mm of mercury. The finding will be discussed below. It was evidently advisable to make as few blood pressure determinations as possible in any one experiment and none was attempted if swelling or edema of the foot appeared.

A comparison of the blood pressure findings obtained by the two methods appears in Tables I to III, which will be discussed below. For the present it will suffice to say that a comparison of the figures in the 4th and 5th columns indicates a remarkably good agreement. From this it is clear that the method for determining blood pressure by observation of the circulation in the claw is adequate for most purposes.

Variations in the Blood Pressure of Mice

Under the circumstances of our studies the blood pressure of the mice differed greatly, and the pressure in individual animals also varied much from time to time. Because of the lack of data on the blood pressure in mice, it seems necessary to present a record of the variations we have encountered, in order that others may know of them who wish to employ the method described.

It is generally known that animals under deep anesthesia have a lower blood pressure than those lightly anesthetized, and furthermore, that blood pressure

TABLE I
The Blood Pressure of Mice Anesthetized with Nembutal

Experiment No.	Time after injection of nembutal min	Stage of anesthesia	Carotid blood pressure mm Hg	Cuff pressure mm Hg	Remarks
1	44	Very light	111	103	
	50	"	116	112	
	60	"	118	113	
	70	"	121	—	
2	50	"	119	114	
	60	"	117	114	
	80	"	120	115	
	35	Deep	85	60	
3	50	Light	96	92	
	70	Very light	101	104	
	90	Light	126	120	
4	93	"	120	115	
	120	"	83	83	
	32	"	103	106	
	59	"	105	105	
5	67	"	102	100	
	74	"	100	98	
	82	"	95	85	Edema appearing in the foot
	92	"	90	87	Edema pronounced in the foot
	107	"	82	72	" "
	117	"	74	64	" "
	142	"	64	53	" "
	147	"	46	37	Animal moribund
	55	Deep	96	94	
	65	"	90	87	
6	80	"	72	68	

The blood pressure measurements from the carotid and by way of the pressure cuff on the leg were made simultaneously

TABLE II

The Blood Pressure of Mice Anesthetized with Nembutal				
Experiment No.	Time after injection of nembutal min	Stage of anesthesia	Carotid blood pressure mm Hg	Cuff pressure mm Hg
7	70	Very light	124	124
8	67	Light	103	—
9	90	Very light	102	100
10	50	"	102	97
11	47	Moderate	100	—
12	70	Light	90	—
13	60	Deep	97	96
14	45	"	86	—
15	80	"	82	78

TABLE III

The Blood Pressure of Mice Anesthetized with Limbinal				
Experiment No.	Time after injection of limbinal min	Stage of anesthesia	Carotid blood pressure mm Hg	Cuff pressure mm Hg
16	90	Deep	96	94
17	45	"	74	68
18	45	"	65	65

The blood pressure measurements from the carotid and by way of the pressure cuff on the leg were made simultaneously

varies greatly with changes in the physiological state. Our findings in the mouse are in accord with this knowledge (Tables I to III).

The physiological state of the animals varied much from instance to instance. In some animals cannulation of the carotid artery was done rapidly and with ease, in others slowly and with difficulty. The trauma of the operation and manipulation of the needle in the tissues of the neck must have much influenced matters. Further, we desired to measure the blood pressure in animals as lightly anesthetized as possible.

TABLE IV

Experiment No	Time after injection of the anesthetic	Blood pressure in the leg "Cuff method"	Comment on depth of anesthesia
	<i>hrs</i>	<i>mm Hg</i>	
1	$\frac{1}{8}$	76	No response on pricking tail deep anesthesia
	$\frac{3}{8}$	84	Response on pricking tail moderate anesthesia
	1	105	Occasional movement of legs light anesthesia
	$1\frac{1}{4}$	109	Frequent movement of legs very light anesthesia, at 1 hr 40 min withdrew leg from cuff and ran off
2	$\frac{1}{4}$	80	Response to pricking tail moderate anesthesia
	$\frac{1}{2}$	82	" " " " " "
	$\frac{3}{4}$	98	" " " " " "
	1	104	Occasional movement of legs light anesthesia
	$1\frac{1}{4}$	106	" " " " " "
	$1\frac{1}{2}$	114	Frequent movement of legs very light anesthesia
	$1\frac{3}{4}$	—	At 1 hr 44 min withdrew leg from cuff and ran off
3	$\frac{1}{3}$	72	No response on pricking tail deep anesthesia
	$\frac{2}{3}$	88	Slight response on pricking tail moderate anesthesia
	$1\frac{1}{4}$	98	Occasional movement of legs light anesthesia
	$1\frac{1}{2}$	118	Frequent movement of legs very light anesthesia, at 1 hr 32 min withdrew leg from cuff and ran off

Blood pressure readings made by the "cuff method" alone in mice anesthetized with nembutal and subjected to no trauma. As anesthesia became lighter the blood pressure rose.

The amount of nembutal given was just sufficient to maintain anesthesia during the average time required for cannulation of the carotid artery. As the time required to perform the cannulation varied much, the initial blood pressure readings were made in some instances as early as 35 minutes after giving the anesthetic (Experiment 3, Table I), while the animals were still deeply under its influence. In other instances, as in Experiments 4 (Table I) and 9 (Table II), the initial blood pressure readings were not obtained until $1\frac{1}{2}$ hours after giving the nembutal, and the animals were in a state of light narcosis rather than anesthesia. Apart from these differences, the response of some of the animals to the uniform dose of nembutal varied much. Some remained deeply anesthetized for periods of approximately $1\frac{1}{2}$ hours, Experiments 6 (Table I)

TABLE V

Experi- ment No	Time after injection of the anesthetic	Blood pressure in the leg Cuff method	Depth of anesthesia	Experi- ment No	Time after injection of the anesthetic	Blood pressure in the leg Cuff method	Depth of anesthesia
	<i>hrs</i>	<i>mm Hg</i>			<i>hrs</i>	<i>mm Hg</i>	
1	1 $\frac{1}{4}$	70	Deep	10	1 $\frac{1}{2}$	91	Moderate
	1 $\frac{3}{4}$	84	Moderate	11	1 $\frac{1}{4}$	87	
	2	87	Light				
	2 $\frac{1}{4}$	91		12	2	90	Light
	2 $\frac{3}{4}$	100			2 $\frac{1}{2}$	103	Very light
	3	106	Very light	13	2 $\frac{1}{4}$	92	Light
2	3 $\frac{1}{2}$	109					
	1 $\frac{1}{2}$	62	Deep	14	1	69	'
	2	62	Moderate		1 $\frac{1}{2}$	68	'
	2 $\frac{1}{2}$	62	"		2	76	
	3	95	Light	15	1	93	Very light
3	3 $\frac{1}{2}$	102	Very light		1 $\frac{1}{4}$	88	'
	1	72	Deep		1 $\frac{3}{4}$	86	
	1 $\frac{1}{4}$	72	'	16	2 $\frac{1}{2}$	93	"
	1 $\frac{3}{4}$	82	Moderate		1	105	'
	2 $\frac{1}{2}$	82	Light		1 $\frac{1}{2}$	112	
4	3	93	Very light		2	111	'
	1 $\frac{1}{2}$	69	Deep		2 $\frac{1}{2}$	114	
	1 $\frac{3}{4}$	76			3	114	
	2 $\frac{1}{2}$	87	Moderate				
5	3	94					
	1	96	Deep				
	1 $\frac{1}{2}$	90					
6	2	72					
	1	81	'				
	1 $\frac{1}{2}$	81					
7	2	92	'				
	2 $\frac{1}{2}$	60					
8	3	62					
	1 $\frac{1}{4}$	78					
9	2	80					
	1 $\frac{3}{4}$	71					

Blood pressure readings made by the cuff method alone in mice anesthetized with luminal and subjected to no trauma. The table shows a correlation between the depth of the anesthesia and the level of blood pressure.

and 15 (Table II) The three animals given luminal (Table III) also remained deeply anesthetized

In all the experiments the depth of anesthesia or narcosis was judged from time to time by the animal's response to a light puff of air into the nostrils or a slight pin prick in the skin of the tail In the tables the depth of anesthesia at the time of each reading has been recorded as "deep," "moderate," "light," or "very light" By deep anesthesia is meant surgical anesthesia with loss of reflexes, by moderate anesthesia a stage in which the animal responded to the stimuli mentioned above by a momentary twitch During light anesthesia mice without direct stimulation made occasional spontaneous movements tending to withdraw the leg from the cuff, and when the state of anesthesia was very light these movements became frequent

In Table I, Experiments 1 to 3 summarize the findings from three of six animals which recovered from the anesthetic sufficiently to withdraw the leg from the pressure cuff a few minutes after the last readings were taken It is clear from this fact that the anesthesia had almost entirely worn off In all instances, the lighter the anesthesia the higher were the blood pressure readings In three other experiments (4 to 6, Table I) the blood pressure fell as time passed The data of Experiment 5 illustrate the fact mentioned earlier, that repeated estimations of blood pressure by the cuff method sometimes led to the development of a discrepancy between the pressure readings taken from the leg and those obtained from the carotid artery In this instance the experiment lasted for $2\frac{1}{2}$ hours, and the animal obviously suffered from shock and exposure Ten measurements of blood pressure in the leg were made, each in duplicate or triplicate The foot showed visible edema after the fourth determination, as indicated in the table, and with the passage of time the edema increased Similar findings appeared in other experiments, which need not be included in the table

Table II gives the data from nine experiments in which single pressure readings only were attempted It will be seen that there was a rough correlation between the level of the blood pressure and the depth of anesthesia when judged as described earlier Finally, Table III shows the blood pressure readings that were obtained from three animals anesthetized with luminal Though all were in the state of deep anesthesia, the first animal $1\frac{1}{2}$ hours after giving the anesthetic showed a higher blood pressure than the others $\frac{3}{4}$ of an hour after receiving luminal

Tables IV and V show well the variations in blood pressure that have been found in mice which were deeply, moderately, or lightly anesthetized with nembutal or luminal In these experiments the animals were not operated upon and they lay quietly on a warming pad with one leg in the pressure cuff In most of the experiments blood pressure determinations were made from time to time while the animals recovered from the anesthetic The three mice of Table IV recovered sufficiently to withdraw the leg from the pressure

cuff and run off. Inspection of Table V shows that in most of the lightly anesthetized mice the blood pressure was relatively high 1 to 2 hours after injecting the anesthetic (Experiments 12 to 16, inclusive). In the animals deeply anesthetized (Experiments 1 to 9) the initial readings were low in comparison with the later ones.

COMMENT

The data of the tables indicate, as might have been expected, that the blood pressure of mice lightly anesthetized with nembutal or luminal was higher than that of animals deeply anesthetized.

Bonsmann (1), studying the blood pressure in the tail of mice by means of a pressure cuff and photoelectric cell, found a 20 to 45 per cent reduction in the pressure after administration of 30 per cent of the fatal dose of chloral hydrate and morphine. The blood pressure of the mouse as reported by him varied from 70 to 100 mm. of Hg, figures lower than those of the present work in which it varied from 60 to 126 mm. of Hg. We attribute the difference to the accuracy of the present method.

SUMMARY

Advantage has been taken of the relative transparency of the claw of the mouse to devise a method, here described, to measure the blood pressure in the animal's leg. Direct measurements of the systolic blood pressure from the carotid arteries of anesthetized mice have also been made. Simultaneous blood pressure readings by both these methods applied to the same animal showed close agreement.

The systolic pressure ranged from 60 to 126 mm. Hg, according to the conditions.

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EXPLANATION OF PLATES

These photographs were made by Mr Joseph B Haulenbeck.

PLATE 4

FIG 1 Apparatus for the determination of blood pressure in the mouse by way of a pressure cuff on the leg and the carotid artery (see text)

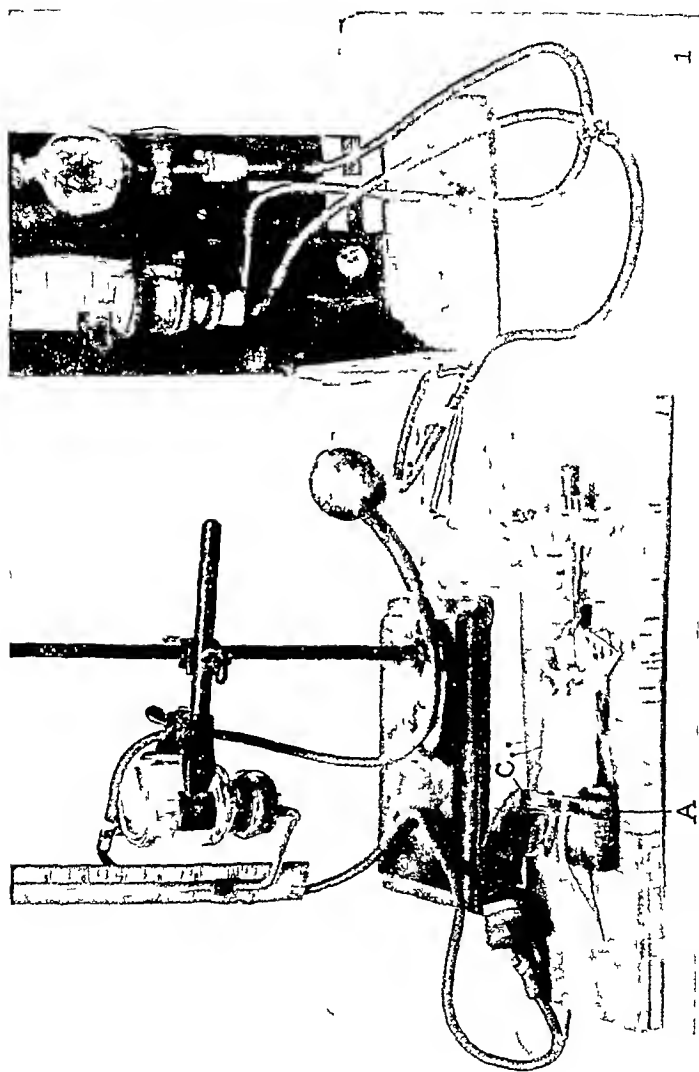


PLATE 5

FIGS 2 and 3 Blood vessels in the claw of the mouse after injection of a gelatin mass containing India ink $\times 100$



(McMaster Peripheral arterial blood pressure in mouse)

THE SWINE LUNGWORM AS A RESERVOIR AND INTERMEDIATE HOST FOR SWINE INFLUENZA VIRUS

I THE PRESENCE OF SWINE INFLUENZA VIRUS IN HEALTHY AND SUSCEPTIBLE PIGS

By RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, March 22, 1941)

In this series of papers data will be presented which demonstrate the fact that the swine lungworm serves under natural conditions as a reservoir and intermediate host for the swine influenza virus. The virus can persist in a masked form within its worm host for long periods of time, and months or even years may elapse between its transmission from one swine to the next. The period during which the virus survives in the lungworm is more than adequate to account for its persistence between epizootics of swine influenza. In this first paper facts will be presented which show that healthy susceptible pigs sometimes contain the swine influenza virus and may undergo attacks of influenza if the virus is provoked to activity by multiple intramuscular injections of the bacterium *Hemophilus influenzae suis* (1).

Of the two agents which act in concert to cause swine influenza (1), it has been shown that the bacterial component, *H. influenzae suis*, is capable of eliciting an immune response that affords only partial protection against the disease (2). Swine influenza virus vaccines, on the other hand, confer a complete immunity to swine influenza (3). The observations now to be recorded were made as a result of further study of the use of *H. influenzae suis* vaccines in the prophylaxis of swine influenza.

Swine Influenza Precipitated by Inoculation with Hemophilus influenzae suis Vaccines

Preparation of H. influenzae suis Vaccines—Cultures 18, 23, and 28 *H. influenzae suis* originally obtained from naturally occurring field cases of swine influenza, were pooled for use in the experiments. The 48 hour growths from potato extract-chocolate agar slants were scraped off and suspended in a small amount of physiological saline. These suspensions were then centrifuged in graduated tubes for $\frac{3}{4}$ hour at 1600 to 1800 R.P.M. The volume of bacterial sediment was noted, after which the sediment was resuspended in sufficient physiological saline to make a final 1 per cent by volume suspension. Part of the suspension was removed to use as living vaccine while the

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remainder was heated at 57°C for 30 minutes in sealed tubes submerged in a water bath. All heated suspensions proved sterile when planted on media capable of supporting the growth of *H influenzae suis*.

Two of the strains used, 18 and 23, had been under cultivation sufficiently long that, while still capable of producing influenza when given intranasally to swine in mixture with swine influenza virus, they no longer transferred with the virus from sick to normal animals by contact (4). Strain 28 on the other hand had been but recently isolated and, with the virus, transferred readily from swine to swine by pen contact.

Source of Experimental Swine Employed—Ordinarily, swine reared on the Institute farm are employed in experimental work. However, at the time that the present experiments were being conducted the supply of swine of our own rearing was limited making it necessary to purchase outside animals for use. 27 of these were obtained from a breeder in whose swine drove swine influenza had never appeared, to his knowledge. In a preliminary experiment 2 of these animals were tested for susceptibility to swine influenza by intranasal inoculation with a mixture of swine influenza virus and the bacterium *H influenzae suis* and found to be fully susceptible. The remainder were bled, and samples of their blood sera tested for the presence of neutralizing antibodies for swine influenza virus. In a serum dilution of 1:2, three serum samples were found capable of partially neutralizing swine influenza virus. These three sera contained sufficient antibody to protect mice against death but not against the production of lesions when the usual neutralization technique was employed (5). This type of finding was different from what might have been expected had the antibodies arisen as the result of previous swine influenza infection, for serum from swine recovered from an attack of swine influenza neutralizes the virus completely in quite high dilution. At the time, the finding of partially neutralizing antibody in the sera of 3 of the animals was tentatively relegated to the vague classification of natural antibody, and the 3 animals supplying the sera were used in experiments other than those under discussion. The remaining swine in the group purchased were considered, on the basis of absence of neutralizing antibody in their sera and the full susceptibility of representative members of the group, not to have had previous experience with swine influenza and to be satisfactory for use in swine influenza experiments. The animals were about 2 months of age when purchased and were kept under observation in semi-isolation for almost 2 months prior to their introduction into the present experiments. They were all found to be infected in varying degrees with ascaris and lungworms, parasites which from past experience were not considered to influence materially the course of a swine influenza infection.

Attempted Vaccination of Swine with Heated and Living H influenzae suis—During December of 1936 each of 4 swine was given three intramuscular injections at 8 day intervals of heat-killed *H influenzae suis*, a second group of 4 swine received injections similarly of living *H influenzae suis*. The amount of the first dose administered was 1 cc, while the two succeeding doses were 2 cc each. No noteworthy reaction was observed in any of the 8 swine following either their first or second injections of vaccine. However, after the third injection, in the cases of all 8 animals, a surprising and puzzling reaction

occurred Since its character varied depending upon whether the animals had received living or heat killed vaccine, the two groups will be discussed separately

Reaction in Swine Vaccinated with Living H influenzae suis—On the 2nd day after the third injection, the temperature of swine 1843 rose to 40.9°C and the animal appeared ill. The following day the animal was prostrated and had labored breathing. By the next day it appeared extremely ill, and it was moribund on the following day. It died on the 4th day after its initial temperature rise and the findings at autopsy were strongly suggestive of fatal swine influenza. 2 other animals swine 1840 and 1847, exhibited temperature elevations to 40.9° and 40.4°C, respectively, on the 3rd day after their third injections of living *H influenzae suis*. Swine 1840 was ill for 6 days with what clinically could not be distinguished from swine influenza. The illness of swine 1847 clinically resembled mild swine influenza and lasted for 4 days. The 4th animal in the group, swine 1844, developed a temperature of 41.2 C on the 4th day after its third injection of *H influenzae suis* and exhibited for 5 days an illness that was clinically indistinguishable from swine influenza.

Reaction in Swine Vaccinated with Heat Killed H influenzae suis—All 4 of the swine injected with heat killed *H influenzae suis* exhibited an extremely mild and indefinite illness for 2 or 3 days, beginning on the 2nd or 3rd day after their third injection. The clinical picture shown by these 4 animals was characteristic of that seen in "filtrate disease" (1) and would probably have entirely escaped notice had not the 4 swine receiving the living *H influenzae suis* vaccine been ill at the same time.

Experiments to Determine the Cause of the Disease Resulting from Multiple Injections of H influenzae suis—Pieces of lung of swine 1843, the animal which had died on the 4th day, were tested for the presence of swine influenza virus by mouse inoculation (6). An agent typical in all respects of swine influenza virus was demonstrated. Blood serum was obtained from the remaining 7 swine following their recovery, and all seven samples neutralized swine influenza virus completely, although failing to exert any effect on the PR8 strain of human influenza virus. Furthermore, the 7 recovered swine were subsequently tested for immunity to swine influenza and found to be fully immune. It thus seemed clear that the reaction observed in all 8 of the experimental animals following their third injection of *H influenzae suis* had as its basis infection with the swine influenza virus. The disease observed in the animals injected with living *H influenzae suis* was true swine influenza in that both the virus and the bacterial component were active, while the disease developing in the animals inoculated with heat killed *H influenzae suis* was "filtrate disease," such as is caused by experimental infection with the swine influenza virus alone (1), and apparently precipitated in the present instance by the inoculation with heated *H influenzae suis*. No explanation of the source of the swine influenza virus responsible for these infections was apparent from consideration of the experiments just discussed.

Confirmation of the Findings

Late in January of 1937 4 more swine were placed in isolation and injected intramuscularly, as in the preceding experiments, with 1 per cent suspensions of heat-killed *H influenzae suis*. On the 3rd day following the second injection this time, 2 of the 4 animals developed temperatures in the neighborhood of 41°C and appeared mildly ill. The other 2 animals appeared mildly ill also, but their temperatures remained within normal limits. One of the febrile swine was killed on the 2nd day of fever and the other one on the 3rd day of fever, and at autopsy the findings in the respiratory tract were characteristic of a filtrate disease more extensive than usual. However, the lesions, instead of being limited to the anterior lobes as is usual in swine infected intranasally with virus, were diffusely scattered throughout the lung and were especially numerous at the bases of the diaphragmatic lobes. Swine influenza virus typical in all respects was demonstrated in both respiratory tracts by mouse inoculation. The 2 afebrile swine were kept under observation. They remained mildly ill for 2 days. They were bled 11 days later, and the serum of each neutralized swine influenza virus completely but was without effect on the PR8 strain of human influenza virus. It seemed clear that the reactions following the second injection in this group of experiments had been due to infection with the swine influenza virus. They thus confirmed the previous observations. The clinical picture exhibited by 2 of the animals was characteristic of filtrate disease, while in the remaining 2 which developed febrile reactions of 41°C the clinical pictures were more severe than is ordinarily seen in swine infected with virus alone. The characteristics of the findings presented at autopsy were, however, typical of an extensive filtrate disease.

With this confirmation of the original observations it seemed that a regularly reproducible phenomenon was being dealt with. The situation, as it appeared from the data available at the time, could be summarized as follows. Apparently normal swine, given multiple intramuscular injections of suspensions of living *H influenzae suis*, developed typical swine influenza in which both *H influenzae suis* and swine influenza virus participated as infective agents. Similar swine given multiple intramuscular injections of heat-killed *H influenzae suis* developed filtrate disease, in which the swine influenza virus was the sole infective agent. In neither set of experiments had swine influenza virus knowingly been introduced, and the origin of the virus infecting the swine was obscure.

Possible Sources of Virus

At the time, four possible sources of the virus were considered, either to be studied further or discarded as impossibilities. These may be briefly summarized as follows.

- 1 The virus might have been present as a contaminant of one of the cultures of *H influenzae suis* used. This possibility could be eliminated on three grounds. First, direct test of the cultures by the intranasal inoculation of swine or mice failed to reveal virus, second, the heat-killed bacterial sus-

pensions had been beaten well above the thermal death point of the virus, and lastly, had virus been present in the bacterial suspensions it should have immunized swine when given intramuscularly rather than induced infection (3)

2 The isolation technique might have been inadequate to prevent accidental infection. This possibility did not seem to furnish a reasonable explanation because at the time the experiments under discussion were conducted there were no cases of swine influenza in the laboratory. Furthermore, the isolation technique employed was the same as that used here for 8 years of more or less continuous investigation of swine influenza without an accidental cross infection.

3 The swine used may have been carriers of swine influenza virus. This possibility was not considered very likely, because at the time no way of introducing swine influenza virus into swine was known that did not cause either infection or the acquisition of immunity. It had been established that virus given intranasally induced infection regularly, while administered by any other route it regularly immunized without causing recognizable infection. Since the swine used in the present experiments proved fully susceptible to infection and their sera were free of neutralizing antibodies, it had been concluded that they had not had a previous experience with swine influenza virus and thus could not be carriers of the virus. The possibility that virus might have gained access to the swine without either infecting or immunizing seemed remote.

4 The virus may have arisen *de novo* as a result of the experimental procedures to which the swine had been submitted. This possibility was included to be considered seriously only in case one of the three preceding was not found applicable.

Attempts to Extend the Observation and to Determine the Nature of the Phenomenon

Further experiments of the type described earlier were carried out in the hope of learning more of the phenomenon and determining the source of the swine influenza virus responsible for the infection that followed multiple injections of *H. influenzae suis*. At this phase of the investigation swine of our own rearing were again available and the supply of those purchased outside and used in the original experiments had been exhausted. Consequently in subsequent experiments our own swine were used. The first of these experiments failed completely to duplicate the original observation. So did the second and the third groups of experiments. Swine were given multiple intramuscular injections at 8 day intervals but remained perfectly normal throughout, neither acquiring swine influenza nor developing antibodies neutralizing swine influenza virus in their sera. As a result of this group of unsuccessful experiments the possibility was considered that the phenomenon

might be more closely related to the source of swine than had been considered likely in the beginning. Because of this, 8 more swine were purchased from the outside breeder who had furnished the original animals. These were of the same stock as purchased before but from later farrowings. After determining that their blood sera were free of swine influenza virus-neutralizing antibodies they were given multiple intramuscular injections of *H influenzae suis*. No illness resulted from a long continued course of injections at 8 day intervals, nor did the animals develop swine influenza virus-neutralizing antibodies. With these failures it seemed apparent that a new attack on the problem was indicated.

Consideration of the experimental factors which might have changed between the time of the earlier positive experiments and the current negative ones suggested *H influenzae suis* itself as probably the most labile. Because of the possibility that the cultures employed might have varied it was decided to obtain some fresh field strains for use. Seven strains were isolated in Iowa from naturally occurring cases of swine influenza. These seven cultures were pooled and administered intramuscularly to swine at 8 day intervals, but they, too, failed to induce a swine influenza virus infection in the experimental animals.

With the apparent exhaustion of the possibility that the source of swine or the cultures of *H influenzae suis* themselves were responsible for the failure to duplicate the original experiments, other possibilities were considered. The original swine had been kept, prior to experimental use, in rather crowded quarters in a pen indoors, and it seemed that this fact might conceivably furnish a clue to the character of their peculiar reactivity to multiple injections of *H influenzae suis*. Because of the crowding, cleaning of the pens had not been as scrupulous as it might have been under less crowded conditions, and it was reasoned from this that more than the usual opportunity had been afforded for the building up of heavy parasitic infections. It was furthermore reasoned, on the possibility that virus might have been made to arise *de novo*, that it would probably have been generated at the intramuscular site of injection of *H influenzae suis*, under which circumstance it would have had to be transported in some way to the susceptible tissues of the respiratory tract. It seemed possible that the failure of the later experiments might have been due to a lack of this hypothetical transporting agent. Because the swine ascaris fitted the picture of a parasite whose larval stage migrated widely throughout the body before eventually becoming established in the gastrointestinal tract, experiments were planned in which wandering ascaris larvae would be present in the animals at the time of their second or third injections of *H influenzae suis*. To this end, swine were fed embryonated swine ascaris ova (7) 2 or 3 days prior to their second or third injections of *H influenzae*

*suus*¹ Usually on the 8th day, occasionally somewhat later, after the ascaris feeding, the animals exhibited clinical signs of respiratory tract involvement. They became depressed, their respiratory rates were accelerated, and their temperatures were elevated to fever level. However, at autopsy the findings in the lung were only those characteristic of an ascaris pneumonia, and swine influenza virus could not be demonstrated in the respiratory tracts. Furthermore, swine that had been treated in this way and allowed to recover failed to develop swine influenza virus neutralizing antibodies in their blood sera upon recovery. It thus seemed evident that the ascaris infestation had not furnished the requisite factor.

A number of other things were tried. Swine were kept in dirty pens, others were underfed, some were kept in cold isolation units, and others were kept in unusually warm isolation units, but under none of these conditions did multiple injections of *H. influenzae suus* exert the slightest effect so far as in inducing a swine influenza virus infection was concerned.

In an accompanying paper experiments which explain the phenomenon will be reported.

SUMMARY

Multiple intramuscular injections of *H. influenzae suus* were found to precipitate swine influenza virus infections in a group of apparently normal swine. The most likely explanation of the phenomenon seemed to be that the animals, though healthy and susceptible, harbored the virus in some unknown manner. The factors possibly determining the phenomenon were explored experimentally but without success.

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¹ I am indebted to Dr. Norman R. Stoll and Dr. G. L. Graham for the advice and help they gave me in handling the parasitological aspects of these experiments.

THE SWINE LUNGWORM AS A RESERVOIR AND INTERMEDIATE HOST FOR SWINE INFLUENZA VIRUS

II THE TRANSMISSION OF SWINE INFLUENZA VIRUS BY THE SWINE LUNGWORM

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In the preceding paper (1) experiments have been described in which swine influenza virus infections were elicited in apparently normal swine by multiple intramuscular injections of suspensions of either living or heat killed *Hemophilus influenzae suis*. The findings suggested that the virus of swine influenza had been present somewhere in the pigs at the time of injection. In the present paper it will be shown that the swine lungworm is capable of harboring swine influenza virus and of transmitting it from swine to swine. This discovery was made incidentally to observations on the possible rôle of the lungworm in transmitting hog cholera.

Study of the possibility that the swine lungworm might serve as an intermediate host for hog cholera had as its basis the popular belief held by certain farmers and veterinarians in the Middle West that the earthworm is responsible, in some unvisualized fashion, for the persistence of cholera virus from one outbreak of the disease to the next.¹ The observations of the Hobmaiers (2) and Schwartz and Alicata (3) that the earthworm serves as intermediate host for the swine lungworm lent some plausibility to this belief, although it seemed more likely, if the earthworm were involved, that its rôle was indirect and that the lungworm in all probability was the actual carrier of the infectious agent. It was decided to test the possibility experimentally. In the hope that findings with one agent might serve as controls for those with another, a study with the swine influenza virus was conducted in parallel with that of the hog cholera virus. The latter yielded negative results whereas that with the influenza virus clearly showed that the lungworm was capable of serving as the transmitting intermediate host. For this reason the findings with the influenza virus will be especially stressed.

¹ I am indebted to Dr. Fred J. Crow and Mr. Ivan Hummer for first calling my attention to this belief.

Materials and Methods

Viruses—The swine influenza virus used was strain 15 obtained originally in Iowa in 1930 from a naturally occurring case of swine influenza. It had been maintained by passage through swine at intervals of 2 months or less.

The strain of hog cholera virus employed had been obtained from a commercial laboratory in 1937. Prior to use in the present experiments it had been maintained by occasional passage through swine at this laboratory.

Earthworms—The earthworms were dug locally in areas known to have been free of contamination by swine feces for at least 10 years. Furthermore, repeated examinations of representative specimens of earthworms from these areas have failed to reveal the presence of swine lungworm larvae. Four species of earthworms,² *Eisema foetida* (Savigny), *Allolobophora caliginosa* f. *typica* (Savigny), *Allolobophora longa* Ude, and *Lumbricus terrestris* Linnaeus, have been utilized in the experiments, and all seem equally capable of serving as intermediate hosts for the swine lungworm.

The earthworms for each experiment were kept in separate wooden barrels of the type in which bulk chemicals are shipped. These barrels, measuring approximately 18 inches in diameter by 30 inches in depth, were buried in the earth to within 2 or 3 inches of their tops and filled with soil to a level which eventually approximated that of the outside dirt level. Each barrel was covered with a fairly snugly fitting wooden lid. Food for the experimental worms consisted of a handful of either yellow corn meal or used coffee grounds, scattered on the dirt surface of the barrels at intervals of 6 weeks to 2 months, and a 2 to 3 inch surface layer of decaying maple and linden leaves which was maintained at all times. This leaf mulch, in addition to furnishing nutriment, also served to maintain the correct degree of moistness in the upper portion of the dirt. Water was sprinkled on the dirt surface at irregular intervals, depending upon weather and season, sufficiently often to maintain the soil in a moist but not wet condition.

Lungworms—In the present experiments two species of lungworms,³ *Metastrongylus elongatus* and *Choerostrongylus pudendolectus*, have been used in mixture, and no attempt has yet been made to work with either species singly. The developmental cycles of these two lungworms are the same. They are concurrently present in swine of the Institute herd having free access to pasture.

The Lungworm Cycle in Earthworms

The swine lungworm, a nematode parasitic in the bronchioles of the bases of the lungs of swine, has been shown by the Hobmaiers (2) and Schwartz and Alicata (3) to pass the first three of its developmental stages in an earthworm. The life cycle in brief is as follows. The embryonated lungworm ovum deposited in the swine respiratory tract by the female lungworm is coughed up, swallowed, and eventually passed in the swine feces. After reaching the soil it is swallowed by an earthworm, in which it hatches as a first-stage larva. It undergoes two further developmental

² I am indebted to Dr. Libbie Hyman, Dr. Grace Pickford, and Dr. Henry Olson for the identifications of the earthworms used.

³ I am indebted to Dr. Norman R. Stoll for identifying the lungworm species used.

stages in the earthworm eventually reaching its third or infective larval stage. In this stage it is capable of infesting swine and has usually become localized either

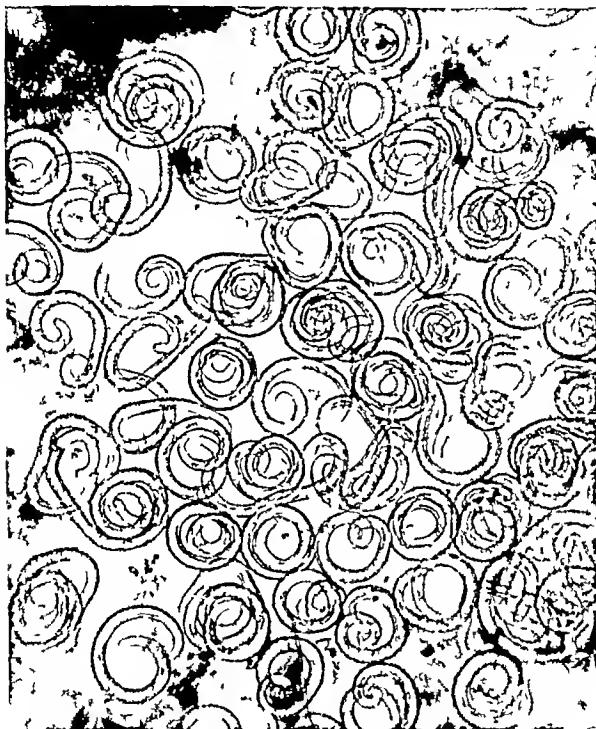


FIG 1 Third stage lungworm larvae as seen in a fresh 'press' preparation of the calciferous gland of an experimentally infested earthworm $\times 94$ Photographed by Mr Julian A Carhle

in the calciferous glands, hearts, or gizzard of its earthworm intermediate host. Examination of fresh 'press' preparations of these organs of an infected earthworm under the low power of the microscope readily reveals the presence of larvae (Fig 1). The larva remains in the third stage until its earthworm host is ingested by a swine. Once within the swine's gastrointestinal tract the larva is liberated, penetrates the swine

intestinal mucosa, and migrates to the respiratory tract by way of the lymphatics and blood stream. It undergoes two further developmental stages in the swine, finally becoming an adult lungworm in the bronchioles at the bases of the diaphragmatic lobes. The whole of the cycle can occupy a span of several years for its completion or, under the most favorable conditions, can be completed in about 2 months.

It is the third larval stage which permits delay in completion of the developmental cycle. In this stage the larva can apparently persist for periods of at least 4 years (4) in its earthworm host.⁴ It is probably because of this very favorable and prolonged survival period in an intermediate host that lungworms constitute such a common parasite in swine reared under the usual farm conditions.

Preliminary Tests

Swine Influenza—Late in October of 1938 lungworms were obtained at autopsy from 3 swine killed on the 3rd, 4th, and 5th days after infection with swine influenza (intranasal inoculation with a mixture of the bacterium *H. influenzae suis* and swine influenza virus) (5). The worms, in a Petri dish containing a little water, were minced coarsely with scissors to release the ova they contained. This worm mince was then buried 3 to 4 inches below the dirt surface in a sunken barrel. Feces collected from the same 3 swine throughout the course of their illnesses together with the colon feces obtained at autopsy were similarly buried in the barrel and loosely mixed with dirt. Shortly afterwards about 400 earthworms were placed on the surface of the soil in the barrel and the dirt was thoroughly wet with a sprinkler of water. The worms promptly burrowed beneath the surface. This barrel was designated as No. 4.

Five weeks later representative earthworms were examined and in their calciferous glands were found third-stage lungworm larvae ranging in numbers from single specimens to as many as 20 or more.

After the lungworm larvae had become established in their earthworm intermediate hosts an attempt was made to learn whether these larvae, hatched from ova obtained from influenza-infected swine, could induce influenza when they infested normal swine. 50 earthworms were removed from the barrel and, after thorough washing in tap water, were fed to 2 swine. In order to be certain that all of the earthworms were consumed they were cut into lengths of a centimeter or less and mixed with the ground grain feed of the swine. The pigs ate this worm-grain mixture with apparent relish.

Hog Cholera—The hog cholera experiment paralleled that with swine influenza both chronologically and in methods used. The lungworms and feces containing lungworm ova were obtained from 2 swine killed when moribund of hog cholera on the 6th and 10th days after infection with hog cholera virus. This material was fed to a similar number of earthworms in another sunken barrel which was designated as No. 3. These earthworms became infested with larval lungworms in numbers com-

⁴ The life span of the earthworm itself, under natural conditions, is unknown. However, under the artificial conditions of captivity specimens of *E. foetida* have lived for as long as 4½ years, *L. terrestris* for as long as 6 years, and *A. longa* for as long as 10¼ years. (Stephenson, J., *The Oligochaeta*, Oxford, The Clarendon Press, 1930, 637.)

parable to the earthworms in the influenza barrel and at 5 weeks 50 were dug and fed to 2 swine as in the influenza experiment

The 2 swine used in each experiment were normal so far as any past experience with either the hog cholera or the swine influenza virus was concerned. However, all 4 animals, for 3 weeks prior to being employed in the earthworm experiments, had received a series of 3 intramuscular inoculations of suspensions of the bacterium *H. influenzae suis* at 8 day intervals. These inoculations had caused no clinical illness in any of the 4 swine, nor was there any reason to suppose that they had altered the susceptibility of the swine to either hog cholera or swine influenza virus. The animals were considered "normal" with respect to the experiments in which they were to be used. However, as later developments demonstrated, the preliminary inoculations with the bacterium *H. influenzae suis* were of particular significance in determining the results obtained.

Swine 2149 and 2215, that had been fed earthworms containing lungworm larvae from hog cholera animals, and swine 2162 and 2217, fed earthworms containing lungworm larvae from swine influenza animals, were observed for a period of 10 days. All remained normal, and the outcome of the experiments was believed to be negative. Rather than destroy the animals at this time it was decided to continue their courses of intramuscular inoculations of suspensions of the bacterium *H. influenzae suis* that had been interrupted at the time of their introduction into the lungworm experiments. Consequently on the 10th day after they had been fed earthworms each swine was inoculated intramuscularly with 2 cc. of a 1 per cent by volume suspension of a 22 hour culture of *H. influenzae suis* grown on potato-chocolate agar slants. The 2 cholera worm animals, swine 2149 and 2215, remained normal throughout a further period of observation of 13 days.

As has been reported briefly in a preliminary publication (6) the 2 influenza worm animals came down with swine influenza.

On the 3rd day after its intramuscular inoculation with *H. influenzae suis* swine 2217 developed a temperature of 40.9 C and appeared ill (Fig. 2). The following day its temperature was still elevated and the clinical signs exhibited were those characteristically seen in swine influenza. The animal remained febrile and ill for 4 days and then underwent an uneventful recovery. Swine 2162 in the same pen remained normal until 5 days after swine 2217 had first become sick and then it too underwent a 4 day illness that was clinically characteristic of mild swine influenza. Serum drawn from each of these 2 animals during convalescence was found to contain antibodies neutralizing swine influenza virus, whereas that drawn prior to the earthworm feeding had been devoid of antibodies. The serum of neither of the swine fed the cholera worms developed antibodies neutralizing swine influenza virus during a similar period of observation.

It seemed apparent from these experiments that the 2 swine fed lungworm larvae from pigs with swine influenza had undergone attacks of typical swine

influenza. However, in the light of subsequent experiments of this type it is probable that only swine 2217 acquired its swine influenza virus directly from the lungworm larvae ingested. The illness of the other animal in the same pen, swine 2162, probably represented an infection acquired by contact with swine 2217. Furthermore the experiments suggested that more than mere transfer of virus by infected lungworm larvae was required to elicit infection. A provocative stimulus or stress was also essential. In the above experiment intramuscular injections of *H. influenzae suis* had provided the provocation.

So far as the single experiment with lungworm larvae from cases of hog cholera was indicative, it did not appear that the hog cholera virus was capable of transmission *via* the lungworm. Because of the promising lead obtained with swine influenza it was decided to concentrate on this phase of the problem and to abandon, for the time, further investigation of hog cholera.

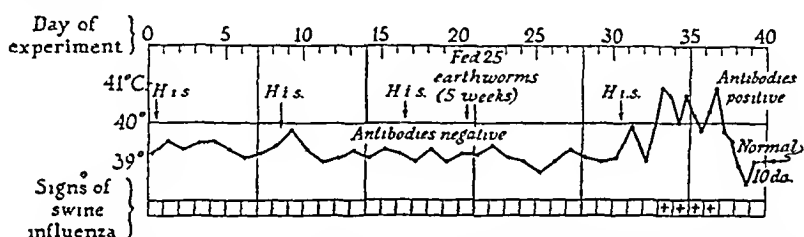


FIG 2 Swine 2217. The animal received three intramuscular injections of *H. influenzae suis* (H_{1s}) before being fed 25 earthworms 5 weeks after these had ingested lungworm ova from swine with swine influenza. Afterwards a fourth injection of *H. influenzae suis* was followed by clinically characteristic influenza. During convalescence virus-neutralizing antibodies appeared in the animal's serum.

Results of Confirmatory Experiments

Experiment 1—In January of 1939, 2 swine, 2200 and 2291, were each fed 18 earthworms removed from barrel 4. By now 2 months had elapsed since the first exposure of these earthworms to lungworm ova from pigs with swine influenza. 19 days after being fed the earthworms each swine was inoculated intramuscularly in the ham with 1 cc of a 1 per cent by volume suspension of a 48 hour potato-chocolate agar live culture of *H. influenzae suis*. The animals remained normal. 8 days later each was given a second intramuscular injection of 2 cc of a 1 per cent suspension of live *H. influenzae suis*.

On the 4th day after this second injection the temperatures of both animals rose abruptly and on the following day they exhibited clinical signs characteristic of swine influenza (Fig 3). Swine 2200 was killed and autopsied on the 3rd day of illness. At autopsy the pathological alterations in the lung were characteristic of those of swine influenza, and swine influenza virus was demonstrated in the lung by mouse inoculation (7, 8). The distribution of the lesions in swine 2200 was somewhat different, however, than that ordinarily seen in intranasally inoculated swine (9). In-

stead of the pneumonia being limited to the cephalic and cardiac lobes as is usually the case, it was rather diffusely distributed in a lobular fashion and portions of all lobes were involved. From past experience with swine infected nasally, extensive involvement of the diaphragmatic lobes except in fatal cases had come to be looked upon as very exceptional. The pneumonia at the extreme bases, in the regions of bronchioles containing adult lungworms was especially marked.

The other animal swine 2291, was ill for 3 days and then underwent an uneventful recovery. Its blood serum, obtained after recovery was found to have developed antibodies neutralizing swine influenza virus.

The demonstration of swine influenza virus in the respiratory tract of one animal during the acute stage of illness and of specific virus neutralizing antibodies in the blood serum of the other after recovery made it evident that the influenza like illness of each had indeed been swine influenza and had had as

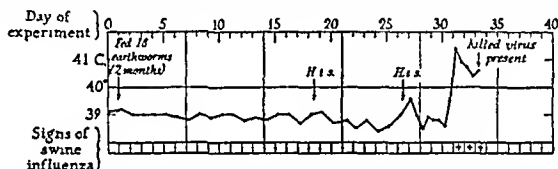


FIG 3 Swine 2200 was fed 18 earthworms which 2 months previously had ingested lungworm ova from swine with swine influenza. 19 and 27 days later the swine was injected intramuscularly with *H. influenzae suis* (H1s). 4 days after the second injection the animal developed swine influenza that was characteristic clinically and at autopsy. Swine influenza virus was demonstrated in the respiratory tract.

its basis infection with the swine influenza virus. The bacterium *H. influenzae suis* was present in cultures from the trachea of the first animal.

Experiment 2—Late in January of 1939 swine 2222 and 2240 were given two intramuscular injections of live *H. influenzae suis* at an 8 day interval. 3 days after the second injection each animal was fed 7 earthworms removed from barrel 4. By now 3 months had elapsed since the first exposure of these earthworms to lungworm ova from pigs with swine influenza. The animals remained normal throughout an 18 day period of observation. Then each was inoculated intramuscularly in the ham with 2 cc. of a 1 per cent suspension of live *H. influenzae suis*.

On the 3rd day after this inoculation the temperature of swine 2222 rose abruptly and the animal appeared ill (Fig 4). The following day its temperature had risen to 41.3°C and the clinical signs were those characteristic of swine influenza. The animal was killed and autopsied and the findings in the respiratory tract were typical of those seen in swine influenza. As in the animal autopsied in the preceding experiment however, the distribution of the pneumonia tended to be more hazy than ordinarily encountered in swine experimentally infected with swine influenza by the

nasal route Swine influenza virus was demonstrated in the pneumonic lung of this animal, and in lungworms taken from bronchi at the bases, by mouse inoculation *H influenzae suis* was present in cultures from the respiratory tract

Swine 2240 became ill on the 6th day after inoculation and the findings at autopsy on the 3rd day of illness were similar to those described for swine 2222 Swine influenza virus was demonstrated in the lung and in lungworms from this animal by mouse inoculation *H influenzae suis* was present in cultures from the trachea In the light of subsequent experience it is likely that swine 2240 acquired its infection by exposure to swine 2222, the first animal of the pair in the pen to sicken

Experiment 3—Late in April of 1939, 5 swine, 2345, 2340, 2339, 2344, and 2341, that had been reared indoors on a concrete floor and were known to be free of lungworms were each fed 15 earthworms from barrel 4 (5 earthworms on each of 3 consecutive days) 6 months had now elapsed since these earthworms had ingested their

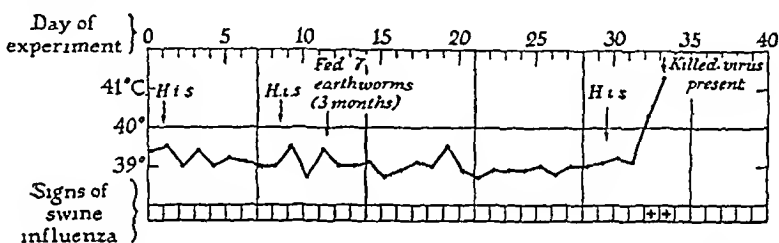


FIG 4 Swine 2222 received two intramuscular injections of *H influenzae suis* (H_{1s}) before being fed 7 earthworms which 3 months previously had ingested lungworm ova from swine with swine influenza The animal remained normal for 18 days and was then again injected intramuscularly with a suspension of *H influenzae suis* On the 3rd day after this injection it came down with swine influenza Virus was demonstrated in its respiratory tract

lungworm ova Swine 2340 was subsequently given three intramuscular injections of suspensions of live *H influenzae suis* at 8 day intervals beginning 13 days after the earthworm feeding It failed to become ill and was killed and autopsied No lungworms could be found upon careful search of its respiratory tract Another of the group, swine 2345, was then killed and autopsied, and its respiratory tract also proved free of lungworms It was thus apparent that lungworms had for some unknown reason failed to become established in this group of swine Consequently in June swine 2339 and 2344 were again fed 18, and swine 2341, 6 earthworms, from barrel 4 Throughout the remainder of the summer they were subjected to inoculations with suspensions of *H influenzae suis* at irregular intervals, but failed to become ill Swine 2344 was finally killed and autopsied late in July Except for the presence of moderate numbers of lungworms in the bronchi at the bases of its diaphragmatic lobes its respiratory tract was normal Furthermore, blood sera from all 3 swine at this time were devoid of virus-neutralizing antibodies Swine 2339 and 2341 were kept under observation and received no further inoculations of *H influenzae suis* until Aug 18 Then each received a series of three intramuscular injections at 8 day intervals

On the 3rd day after the third injection of *H. influenzae suis* the temperature of swine 2341 rose abruptly to 40.9°C and the animal appeared ill. The following day the clinical signs were those characteristic of a mild swine influenza. By the next day the temperature had dropped to normal and the animal appeared to be recovering. It was killed and autopsied and the pathological findings in the respiratory tract were those of swine influenza. Again, however, the pneumonia was predominantly basilar instead of being limited to the anterior lobes as is usual in swine infected by way of the nose. Lungworms were numerous in the bronchi at the bases of the lung.

Eight anesthetized mice were inoculated intranasally in the usual fashion (8) with a 10 per cent suspension of pneumonic lung of swine 2341 in order to test for the presence of swine influenza virus. 4 of these were killed and autopsied on the 4th day. Their lungs appeared completely normal. The lungs of these mice were ground in saline to make a 5 per cent suspension and this suspension was administered intranasally to 8 more anesthetized mice. Of these, 5 died and 3 were killed, and all showed lung lesions characteristic of those caused by swine influenza virus. Furthermore in the next serial passage, mice which received a suspension of lungs of these 2nd passage mice mixed with serum known to neutralize swine influenza virus were completely protected whereas the control mice all died. The remaining 4 mice that had received the original lung suspension from swine 2341 were tested for immunity to a fully mouse adapted swine influenza virus 25 days later. One of these died and the other 3 survived.

It seemed apparent from these experiments that the lung of swine 2341 had contained swine influenza virus. The failure of this virus to kill, as most swine influenza viruses typically do, in its first mouse passage, suggested that its mouse pathogenicity may have been altered in some way either by its sojourn of 8 months in lungworm larvae in their earthworm intermediate hosts or its survival of almost 3 months in the respiratory tract of swine 2341 prior to causing illness.

The remaining animal in Experiment 3, swine 2339, developed swine influenza 2 days after swine 2341 became ill in the same pen. It is believed that this constituted a contact infection. This animal was only moderately ill. Its blood serum, obtained 10 days after infection, neutralized swine influenza virus.

Experiment 4 —In October of 1939 swine 2428 and 2432 known to be free of lung worms, were each fed 12 earthworms from barrel 4 (4 earthworms on each of 3 consecutive days). It had now been a year since these earthworms had ingested their lungworm ova. Beginning 14 days after their earthworm feeding each animal received four intramuscular injections of suspensions of live *H. influenzae suis* at 8 day intervals. The first of these injections in each case was 1 cc. of a 1 per cent suspension of 48 hour potato-chocolate agar cultures while subsequent injections were of 2 cc. Midway between the third and fourth *H. influenzae suis* inoculation each animal had received 10 cc. of a 5 per cent solution of calcium chloride into the right lung and

pleura through the chest wall, a procedure which was known, from experiments to be reported later, to elicit swine influenza virus infections sometimes in swine that had ingested lungworm larvae from influenza swine. The use of calcium chloride to provoke swine influenza virus infections had been suggested originally by the finding of Bullock and Cramer (10) that this substance would break the dormancy of spores of the bacteria of gas gangrene and tetanus in mice or guinea pigs.

Neither animal was noted to be clinically ill as a result of any of the procedures to which it had been subjected and the experiments were considered negative. Swine 2432 was killed and autopsied 5 days after its last injection of *H influenzae suis*. Its respiratory tract was negative except for the presence of dense fibrous adhesions in the right pleura resulting from the calcium chloride injection. There were also numerous adult lungworms in the bronchi of the bases of the diaphragmatic lobes.

Blood serum obtained at autopsy was saved to test for the possible presence of antibodies neutralizing swine influenza virus, a procedure regularly followed in this work. Surprisingly enough in view of the fact that the animal had at no time been clinically ill, its serum neutralized swine influenza virus to a dilution of 1 in 8. This titer was lower than that ordinarily encountered in the sera of convalescent swine (11). However, since the serum of the animal drawn 6 days after its earthworm feeding was free of virus-neutralizing antibodies the presence of antibodies later on must be interpreted as having resulted from an experience with the swine influenza virus.

Since this experience did not result in clinically recognizable illness there is no way of knowing which of the several procedures applied to the animal had elicited the virus response.

The other animal in the pen, swine 2428, failed to become ill, nor did virus-neutralizing antibodies appear in its blood serum in spite of the continuation of efforts to elicit a swine influenza infection by multiple intramuscular inoculations of *H influenzae suis*. When this animal was finally killed and autopsied its respiratory tract was normal except for the presence of numerous adult lungworms in the bronchi of the bases of the diaphragmatic lobes.

In this experiment, then, not only did swine 2432 undergo an immune response to swine influenza virus, but it failed to transmit its virus by contact to swine 2428 in the same pen. The failure of swine influenza virus to transfer by pen contact is of exceedingly rare occurrence in swine that have been infected nasally.

Experiment 5—In March of 1940 swine 2433, known to be free of lungworms, was injected intramuscularly with 1 cc of a 1 per cent suspension of a 48 hour potato-chocolate agar culture of *H influenzae suis*. On each of the 2 succeeding days the animal was fed 5 earthworms from barrel 4. 16½ months had now elapsed since these earthworms had ingested lungworm ova from pigs with swine influenza. 11 and 19 days after the earthworm feeding the pig was inoculated intramuscularly with 2 cc of a 1 per cent suspension of live *H influenzae suis*. No illness resulted from either injection. 4 days later, because of a suggestion furnished by Taylor's (12) observa-

tion that sterile fluid into the respiratory tracts raised the influenza virus titer of sublethally infected mice, 15 cc. of broth was administered intratracheally under chloroform-ether anesthesia. This was followed in 2 days by the intramuscular injection of 1 cc. of a 1 per cent suspension of living *H. influenzae suis*.

On the 2nd day after this last injection the animal appeared ill, and its temperature rose abruptly to 41°C. It was killed and autopsied on this 1st day of illness. The findings at autopsy were unusual in that the cephalic, cardiac, and azygos lobes were free of lesions. There was, however, a scattered lobular atelectatic pneumonia of the upper portion of the right diaphragmatic lobe, and the bases of both diaphragmatic lobes were consolidated. There were numerous small but mature (embryonated ova present in the females) lungworms in the bronchi at the bases.

In this instance swine influenza virus of average pathogenicity for mice was demonstrated in the respiratory tract. In its first passage it killed all mice inoculated within 8 days and in the second mouse passage killed all within 5 days. The finding of virus that was fully pathogenic for mice in this pig eliminated from consideration the possibility that the apparent attenuation of the virus for mice in the case of swine 2341 (Experiment 3) had resulted from its 8 month sojourn in lungworm larvae. Here in the case of swine 2433 virus fully pathogenic for mice had been recovered from an animal infected with virus that had survived for 16½ months in lungworm larvae.

Swine 2433 transmitted influenza, by exposure, to another pig in the same pen, and antibodies neutralizing swine influenza virus appeared in the serum of this contact animal during convalescence.

Experiment 6—In November of 1940 swine 2609 and 2629, known to be free of lungworms, were each fed 5, 6, and 10 earthworms from barrel 4 on 3 consecutive days. 2 years had now elapsed since these earthworms had ingested lungworm ova from pigs with swine influenza. Beginning 9 days after their earthworm feeding the pigs were given three intramuscular inoculations of suspensions of live *H. influenzae suis* at 8 day intervals.

No clinically recognizable illness resulted. However, blood serum obtained from both swine 22 days after their third inoculation with *H. influenzae suis* was found to neutralize swine influenza virus. The neutralizing titer of the serum was 1:20 in the case of 2609 and slightly less in the case of 2629 when tested against the usual 1000 M.L.D.s of swine influenza virus. Serum of each swine obtained at the beginning of the experiment had been free of neutralizing antibodies. The serum antibody titer attained was thus lower than that ordinarily encountered in swine convalescent from actual infection (11). It more nearly approached that of swine immunized by virus administered subcutaneously or intramuscularly.

Because both swine remained clinically normal throughout the experiment it is not possible to know which of the provocative inoculations activated the virus. However, the antibody response indicated that each swine had undergone an experience with the swine influenza virus.

Failure to Demonstrate Swine Influenza Virus in Larval or Adult Lungworms

Consideration of the courses of the experiments just outlined and of the procedures required to elicit swine influenza infections made it evident that the swine influenza virus contained in the lungworms must not have been in a readily available or infective form. If fully infective virus were present within lungworm larvae one would anticipate that a swine influenza infection would result as soon as the larvae reached the highly susceptible tissues of the swine respiratory tract, that is to say on from the 3rd to the 8th day after ingestion. Since infections failed to occur then and infested swine remained normal for long periods of time, even after the larvae had developed to adult lungworms, it seemed obvious either that the virus in the worms was present in a masked, non-infective form or that it was not liberated from within the infected lungworm cells until some unusual stress was applied.

A number of experiments have been conducted in an attempt to demonstrate swine influenza virus within larval or adult lungworms by direct or indirect means. Since these have so far been uniformly negative only the more general aspects of the procedures tried will be outlined.

Efforts were first made to demonstrate virus in third-stage larvae.

Calciferous glands and hearts, rich in third-stage lungworm larvae, were removed from earthworms of known influenza-producing capability. These were ground with sterile sand, and suspended in saline. In such suspensions the larvae were thoroughly disintegrated. The suspensions were then administered intranasally to either 6 or 8 anesthetized mice in each experiment. Half of the mice were killed and autopsied on the 4th day after inoculation, and suspensions of their lungs were passed intranasally to a second group of anesthetized mice. This procedure was continued through either three or four serial passages before concluding that swine influenza virus was non-detectable.

In no case were pulmonary lesions, suggestive of those caused by swine influenza virus, observed. 2 or 3 weeks later, the surviving half of the mice through which the material had been passaged were inoculated intranasally with swine influenza virus. All succumbed typically, indicating that no immunity had been conferred by the lungworm larvae suspensions.

In addition suspensions of lungworm larvae similar to those administered to mice were mixed with cultures of *H. influenzae suis* and inoculated intranasally, intratracheally, or directly into the lungs of swine. The animals failed to come down with swine influenza nor did they develop in their sera antibodies neutralizing swine influenza virus. From such experiments it seemed apparent that virus in the lungworm larvae was present either in subinfective titer or in a non-infective or thoroughly masked form.

Efforts were next made to demonstrate virus in adult lungworms removed from the respiratory tracts of swine thought to be "ripe" for provocation of influenza.

The swine furnishing these lungworms had been fed earthworms just as in the experiments outlined earlier. However, they were not submitted to provocative inoculations with *H. influenzae suis* but instead were killed and autopsied a month or longer after their earthworm feeding. The adult lungworms contained in their respiratory tracts were removed, ground with sand, suspended in saline, and administered to mice and swine in a manner similar to that used in testing the larvae for virus.

Neither mice nor swine became infected or developed immunity, and it was necessary to conclude from the tests that swine influenza virus was not demonstrable in the adult lungworms.

An attempt was next made to demonstrate the presence of virus in adult lungworms from "ripe" swine by indirect means. Though the virus was masked and non-infective upon direct introduction into the respiratory tracts of susceptible hosts it yet might prove detectable by means that had been successfully used to demonstrate the presence of masked virus in another disease, rabbit papillomatosis. Here, although suspensions of the papillomas of domestic rabbits are usually non-infectious due to masking (13) of the virus they contain, they will immunize other rabbits to the virus and will even elicit low titer virus-neutralizing antibodies (14) if administered intraperitoneally.

Applying the same general procedure used in detecting masked papilloma virus mice were given repeated intraperitoneal injections of suspensions of lungworms from "ripe" swine. 2 weeks after their last injection they were inoculated intranasally, while etherized, with from 100 to 1000 M.L.D.s of swine influenza virus. All succumbed typically and no immunity was demonstrable.

It must be concluded from the experiments conducted so far that swine influenza virus is not detectable by either direct or indirect means in lungworms known from other evidence to be acting as intermediate hosts for the virus.

DISCUSSION

The experiments described were undertaken in an effort to learn whether the swine lungworm could serve as intermediate host for either the hog cholera or the swine influenza virus. It had been anticipated, if the lungworm were to fit the rôle of intermediate host for either virus tried, that it would transmit the causative agent from sick to normal animals directly. In view of this preconceived notion that the lungworm should fit the general pattern of other known intermediate hosts, the first experiment tried in the case of each virus was considered negative when, after a 10 day period of observation, the animals to which lungworms suspected of carrying virus had been administered, remained normal. 10 days was well beyond the usual incubation period for either hog cholera or swine influenza and furthermore within this period the lungworm larvae should have largely completed their migrations within the host and have become established in the swine respiratory tract. The pos-

sibility that either virus might have been transmitted in a latent, masked, or non-infective form was not considered until, in the course of subsequent events, the findings were such as to make that possibility quite obvious. Because the first experiment with hog cholera virus was negative, further work with it was discontinued and only the more promising experiments with swine influenza were carried further.

It was found that the suggestion furnished in the first swine influenza experiment, that virus transmitted by the lungworm had to be activated in some way to elicit infection, was indeed correct. Swine that had been fed earthworms containing lungworm larvae hatched from ova coming from influenza-infected swine remained normal to all appearances so long as they were kept under the usual experimental conditions. Only when they were subjected to a stress of some unusual character did they develop influenza. The administration of multiple intramuscular injections of suspensions of the bacterium *H. influenzae suis* constituted a satisfactory means of furnishing the required stress. It is probably significant that in no case did influenza follow a single injection of the bacterial suspension. Sometimes it followed the second or third injection, but not infrequently a larger series had to be employed. It is believed that these findings indicate that the provocative stimulus responsible for eliciting the swine influenza infections was not *H. influenzae suis per se* but rather some condition or chain of conditions established by repeated injection of the bacterium. Conceivably it partook of the character of an allergic or sensitization phenomenon.

After infection with the virus was provoked, however, the presence of *H. influenzae suis* was important in determining the character of the resulting disease. In all of the experiments in which clinical illness ensued, *H. influenzae suis* administered intramuscularly had reached the respiratory tract so that, upon activation of the virus, true swine influenza, having as its cause the concerted activity of *H. influenzae suis* and swine influenza virus (5), resulted. In some experiments in which many injections of *H. influenzae suis* had been administered before the virus infection was finally provoked, the resulting swine influenza was milder than usual, probably because of the development of some degree of immunity to *H. influenzae suis* (15). (The case of swine 2341, Experiment 3, illustrates this.) In other experiments which have been carried out but which will not be reported until later, provocation by means other than the use of live *H. influenzae suis* yields filtrate disease, an infection in which only the virus participates.

The experiments reported, all conducted with earthworms taken from barrel 4, give a false impression of the ease and regularity with which the phenomenon under discussion can be reproduced. To date, a total of 69 experiments, containing from 1 to 8 swine per experiment, have been conducted. Of these, 41 have either proved negative or been discontinued before infections were

provoked. The remaining 28 experiments, including those cited in the present paper, have been positive. There are numerous reasons for this only partial success. Undoubtedly some of the failures resulted from the use of lungworms that, for a variety of reasons to be discussed in a later paper, had not become carriers of swine influenza virus. In most of the negative experiments, however, the failures are believed to have been due to the inability to establish, in the experimental swine, appropriate conditions for the virus infection. Only one of the reasons for failures in this category is apparent from the experiments cited, and only this one will be discussed.

The swine in experiment 3 were fed late in April earthworms known to contain lungworm larvae. Because lungworms failed to become established the procedure was repeated in June. From then until September efforts to provoke influenza infections in these animals were unsuccessful. In September, after having proved refractory throughout the summer, one of the animals finally developed swine influenza following a long series of provocative inoculations. In other experiments not included among those dealt with in this paper a similar summer refractory state has been encountered. During 1939 no experiments were successful between April and September, while in 1940 all experiments carried out from May to October were negative. It would seem from such results during two summers that, if swine are not completely refractory during this time of the year, their infections are at least much more difficult to elicit using the same means of provoking that have been successfully used during the autumn, winter, and spring months. This failure of the virus to cause infection by way of its lungworm intermediate host during the summer has, as yet, no explanation. The finding, however, fits well with the known seasonal incidence of swine influenza under field conditions. The result obtained in Experiment 3 indicates, so far as it goes, that the refractory state is due not to the inability of the worm intermediate host to transmit its masked virus during the summer but rather to failure of the provocative stimuli applied to render the masked virus infective during this season of the year. While a number of the 41 negative experiments may have resulted in failure because they were conducted during the summer refractory state, others carried out during known favorable seasons have also resulted negatively. The question of the rôle played by seasonal and other factors in determining the transmission of swine influenza virus by way of a lungworm intermediate host will be considered in detail in a later paper when experiments other than those included in the present paper have been described.

The inability to detect swine influenza virus by direct means either in lungworm larvae in their earthworm intermediate hosts or in adult lungworms removed from the respiratory tracts of swine thought ripe for provocation of the disease has been an interesting but bothersome handicap to the work. While the non-infectiousness of the virus in its intermediate host was to be

anticipated from the failure of infected lungworms to induce disease directly in the swine whose respiratory tracts they infested, it had been hoped that there might be some way of demonstrating its presence by indirect means. Thus far all attempts to do this have been unsuccessful and it is necessary to conclude that the swine influenza virus is in a completely masked non-infective form in its lungworm intermediate host. It evidently remains in this form until unmasked by some stress applied either to the lungworm or to the swine harboring it. This phenomenon of provoking the infectivity of masked swine influenza virus is reminiscent of those experiments of Bullock and Cramer (10) in which the dormancy of spores of the bacteria of gas gangrene or of tetanus in mice or guinea pigs was broken by injecting calcium chloride, a phenomenon which was termed *kataphylaxis*. In like manner, Turner (16) has demonstrated in Black Disease of sheep that spores of *Bacillus oedematiens*, the causative bacterium, may be present in a latent state in the livers of sheep for long periods of time without causing disease. Only when a kataphylactic agent invades the liver, in this case the liver fluke *Fasciola hepatica*, does Black Disease ensue.

In order to demonstrate the presence of swine influenza virus in its lungworm intermediate host it is necessary that the lungworm go through its complete cycle beginning in the respiratory tract of an infected swine and ending in the respiratory tract of a susceptible and properly prepared swine. Fully infective virus is detectable only at either end of such a cycle. Stanley (17) has recently compared this phenomenon very aptly with that of a train passing through a tunnel. One can see the train as it enters and as it leaves, but it is more apparent while in the tunnel than is swine influenza virus while in its intermediate host.

Nothing to indicate that swine influenza virus is injurious to its lungworm host has been observed. Neither is there any evidence that lungworms in the swine respiratory tract are injured by the provocative stimuli applied in eliciting virus infections.

These experiments with swine influenza are not the first instance in which it has proved difficult or impossible to detect the presence of an infectious agent during the time that it is in an intermediate or transmitting host, though it is, so far as is known, the first instance in the case of a filtrable virus. In salmon poisoning of dogs, the non-filtrable causative agent, whose nature remains obscure, is transmitted by the fluke *Nanophyetus salminala*. Though the etiological agent is demonstrable, by dog inoculation, in the encysted cercariae of the fluke in its fish host it is not similarly detectable in the rediae or cercariae of the fluke in the snail host (18). Furthermore in blackhead of turkeys, a disease in which the causative histomonad (*Histomonas meleagridis*) is transmitted by the cecal worm *Heterakis gallinae*, histomonads have not been demonstrated by direct means in *Heterakis* ova (19). Stanley's tunnel simile is as

applicable to the histomonad of blackhead as it is to the virus of swine influenza. If agents having the character of a histomonad or of the non filtrable agent responsible for salmon poisoning are not, by any available means, demonstrable in their intermediate hosts, it is perhaps not surprising that a filtrable virus should be "lost" in its transmitting host. The occult nature of infectious agents within helminth intermediate hosts may prove to be a characteristic of this mechanism of disease transmission. Neither in salmon poisoning nor in blackhead, however, does it seem necessary to provoke or activate the infective agent in order to elicit infection.

The results obtained in the experiments here described may be classified into two groups, depending upon whether or not the experimental swine became clinically ill. In the first group, comprising the original and confirmatory Experiments 1, 2, 3, and 5, the animals developed clinically characteristic swine influenza. The only feature of the disease that differed notably from that seen in intranasally infected swine concerned the distribution of the influenzal pneumonia. In swine receiving their infections experimentally by way of the nose the pneumonia is predominantly localized in the cephalic, cardiac, and azygos lobes with little or no involvement of the diaphragmatic lobes except in cases with a fatal outcome while in swine receiving their infections through the medium of lungworms the pneumonia was more diffusely distributed throughout the lung and the diaphragmatic lobes especially were involved. In the case of swine 2433 (Experiment 5), killed and autopsied on its first day of illness, only the diaphragmatic lobes were involved and all five of the anterior lobes were completely free of lesions. It is believed, on the basis of the autopsy findings in other swine that were permitted to live through 3 or 4 days of illness and in which the anterior lobes were also involved, that the pneumonia in the diaphragmatic lobes probably represented the initial observable lesion in swine acquiring their virus by way of lungworms. Lungworms are localized almost exclusively in the small bronchi at the bases of the diaphragmatic lobes. From these posterior lobes the infection doubtless spreads, probably by way of the bronchi, eventually involving portions of some or all of the anterior lobes.

Of the second group into which the results fall, were those animals that became immune to swine influenza virus without having exhibited clinical evidence of infection—the animals in Experiments 4 and 6. It is possible that these swine may have undergone attacks of filtrate disease too mild for recognition, but this seems unlikely for two reasons. First, the virus neutralizing antibody titers were extremely low to represent the result of frank infections even with the swine influenza virus alone, and second, swine 2432 failed to infect by contact swine 2428 in the same pen, a failure that is extremely rare in either swine influenza or filtrate disease. Neither of these findings alone would necessarily eliminate the possibility that the swine in Experiments 4

and 6 had undergone very mild attacks of filtrate disease, but the two considered together make this possibility seem quite unlikely

The results are more suggestive of those attained in immunizing swine by the subcutaneous or intramuscular administration of virus (20, 21). Such swine develop low titer antibodies and become immune but the virus does not reach the respiratory tract in infective quantities nor spread to other swine by contact from the immunized animals. Such an explanation applied to the results obtained in Experiments 4 and 6 would take into consideration the well known helminthological fact that, among nematode larvae which undergo extensive wanderings in the host's body before reaching their sites of predilection, many are destroyed, or wander into tissues from which they cannot escape. It is believed that the findings with the swine that developed immunity instead of infection can best be explained upon the basis of the activation of virus within lungworm larvae that had been "lost" in non-respiratory tract tissues. There is no reason to suspect, if masked swine influenza virus activated within the respiratory tract results in clinical infection, that that activated outside the respiratory tract should not, like active virus placed there by inoculation, induce an immune response without clinical infection.

It has not as yet been possible to explain the change which takes place in active swine influenza virus when, upon entering the lungworm, it becomes masked. Neither has it been possible to visualize the mechanism whereby the masked virus can again be converted into an infective form upon its return to susceptible swine. That the phenomenon, whatever its mechanism, achieves and insures prolonged survival of the virus is indicated by Experiment 5, in which 17 months elapsed between infection of the swine initially furnishing the virus and the eventual establishment of the virus as an infectious agent in another swine. In like manner, in Experiment 6, 2 years intervened between the swine that originally supplied the virus and the ones that were eventually immunized by it. The swine influenza virus is not to be considered, from its known properties, an unusually resistant agent. It is destroyed in 24 hours by incubation at 37°C in saline suspension, preserved under favorable conditions in 50 per cent glycerol at refrigerator temperature one cannot depend upon its remaining viable for much longer than 2 months, and in the respiratory tract of an infected swine the virus is not detectable after the 7th day. It is thus apparent that the survival periods recorded for swine influenza virus in the present experiments are very unusual and probably could be duplicated under no set of experimental conditions for storage that might be devised. To the bacteriologically trained person, the possibility that masking of swine influenza virus may be comparable in at least some of its characteristics to spore formation among certain bacteria comes to mind at once. Whatever its nature, the mechanism furnishes a potential means for the preservation of swine influenza virus from one epizootic of swine influenza to the next. In fact

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the period of survival of the virus recorded in the present experiments is over twice that which would account for its persistence throughout the usual in terepizootic period

It is believed that the puzzling findings described in the preceding paper (1), in which swine influenza virus infections were elicited in apparently normal swine receiving multiple intramuscular injections of *H influenzae suis*, can be explained on the basis of the results recorded in the present paper. The swine used in those experiments were observed to have been infested with lungworms. From the way in which those experiments and the ones described in the present paper duplicated one another it appears obvious that the same explanation will hold for both. On this basis masked swine influenza virus present in the lungworms of the swine used is considered to have been accountable for the influenza infections induced.

SUMMARY

1 The swine lungworm can serve as intermediate host in transmitting swine influenza virus to swine. The virus is present in a masked non infective form in the lungworm, however, and, to induce infection, must be rendered active by the application of a provocative stimulus to the swine it infests. Multiple intramuscular injections of *H influenzae suis* furnish a means of provoking infection. Swine influenza infections can be provoked in properly prepared swine during the autumn, winter, and spring, but not during the summer. The phenomenon, while not regularly reproducible, occurs in well over half the experiments conducted outside the refractory period of summer. No explanation for the failures is apparent.

2 The virus can persist in its lungworm intermediate host for at least 2 years.

3 Swine infected with swine influenza virus by way of the lungworm intermediate host exhibit a more pronounced pneumonia of the posterior lobes of the lung than do animals infected intranasally with virus. The situation of the worms providing the virus will account for this.

4 Occasional swine infested with lungworms carrying influenza virus fail to become clinically ill after provocation but instead become immune. In these it is believed that lungworms containing the virus are localized outside the respiratory tract at the time of provocation.

5 It is believed that the experiments described furnish an explanation for the findings recorded in the preceding paper, in which swine influenza virus infections were induced in apparently normal swine by multiple injections of *H influenzae suis*.

6 In a single experiment swine lungworms failed to transmit bog cholera virus.

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CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

IV DEMONSTRATION OF COPPER IN THE PURIFIED VIRUS

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PLATE 6

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Previous studies have indicated that preparations of elementary bodies of vaccinia can be obtained which exhibit great constancy in their immunological, physical, and chemical properties. These studies have shown that the virus is composed chiefly of protein, thymonucleic acid, lipid, and carbohydrate, which occur in constant proportions in different lots of purified virus, and in concentrations not materially different from those found in bacterial cells (1, 2). No independent metabolism of elementary bodies has yet been discovered. Parker and Smythe (3), in 1935, were unable to demonstrate the utilization of oxygen by purified elementary bodies in the presence of hexose-monophosphate and "respiratory factor," nor could they obtain evidence of acid production under anaerobic conditions with glucose-monophosphate, bicarbonate buffer, and an extract of tissue as a source of respiratory supplement.

The obligate parasitic nature of the elementary body of vaccinia makes it unlikely that an independent metabolism, apart from the host cell, exists. It is not unreasonable to assume, however, that the elementary body possesses an incomplete metabolic system, relying in part, or even in the main, on constituents within the host cell for its completion. With this hypothesis in mind we have searched for the presence in purified virus of substances which, in bacteria and more highly organized cells, are known to participate in oxidation reduction chains. In this paper results of our search are recorded.

EXPERIMENTAL

A detailed description of the methods used in the concentration and purification of elementary bodies of vaccinia has appeared in an earlier publication (1). Freshly prepared, active virus dried to constant weight, was used in each experiment recorded in this study.

Attempts to Demonstrate a Cytochrome System

Respiration in aerobic organisms is known to go in part, if not chiefly, through the cytochrome system, which acts as a reversible oxidation reduction

link between oxygen and certain enzyme systems which have become reduced in the process of the oxidation of cell substrates (4, 5) So far as it is now known, all cells which are able to use molecular oxygen contain one or more members of a group of protein porphyrins with iron in tetra-pyrrolic combination These substances are known collectively as the cytochromes, and have been shown to participate in reversible oxidation-reduction processes within the cell (5) An enzyme, cytochrome oxidase, concerned with the rapid oxidation of reduced cytochrome, has been studied in detail by Keilin and Hartree (6) Both spectroscopic and enzymatic methods are available for the detection of cytochrome *c* (7)

Spectroscopic Examination—In selected biological material, suitably prepared, the bands of reduced cytochrome can often be seen clearly with the aid of a microspectroscope A search for the cytochrome system in vaccine virus was first made spectroscopically by means of a small Zeiss microspectroscope which was substituted for the eyepiece of an ordinary monocular microscope A hanging drop preparation, containing a thick suspension of elementary bodies in 5 per cent sodium hyposulfite was prepared and examined repeatedly with a strong light source for the bands of reduced cytochrome No absorption of any type was evident within the visible range of the spectrum Under the same conditions the bands of reduced cytochrome in a suspension of yeast were clearly observed The opacity of the elementary body preparation, however, made it possible that faint bands of reduced cytochrome might well have been obscured, and consequently missed by this technique We accordingly turned to the second method, employing cytochrome *c*, cytochrome oxidase, and a variety of hydrogen donors, in an attempt to demonstrate cytochrome oxidase and cytochrome *c*, respectively, in preparations of elementary bodies of vaccinia

Examination of Virus for Cytochrome Oxidase—Cytochrome *c* was prepared by the method of Keilin and Hartree (8) This substance, together with paraphenylenediamine and certain other hydrogen donors, can be used to demonstrate the presence of cytochrome oxidase in suitably prepared biological materials, as shown by oxygen uptake in the Warburg apparatus Under carefully controlled conditions, the rate of oxidation of the paraphenylenediamine in the presence of pure cytochrome *c* is proportional to the concentration of the cytochrome oxidase which is contained in the substance tested

1000 gm of fresh beef heart muscle were freed from fat and ligaments and ground finely in a meat chopper The pulp was mixed with an equal volume of 0.15 N (2.5 per cent) trichloroacetic acid The mixture was allowed to stand at room temperature for 2 hours with occasional stirring, after which the fluid was pressed out, brought to pH 7 with sodium hydroxide, and centrifuged for 10 minutes The clear supernatant fluid was drawn off by suction, and 50 gm of ammonium sulfate for each 100 cc of material was added The precipitate was filtered off and discarded The filtrate

was next treated with an additional 5 gm of ammonium sulfate per 100 cc. and allowed to stand overnight in the cold. The material was then filtered, and while cold there was added 1/40 volume of cold 20 per cent trichloroacetic acid. The fine red precipitate which appeared at this point in the procedure was collected by centrifugation. The precipitate was shaken with saturated ammonium sulfate and centrifuged again, after which it was transferred to a cellophane sac in a minimal amount of distilled water and dialyzed for 48 hours at 4°C against 1 per cent sodium chloride. Finally the contents of the sac were shaken with a few drops of chloroform, filtered, and frozen and dried *in vacuo*.

An analysis performed on a sample of this material, dried to constant weight and ashed, revealed an iron content of 0.4 per cent which is close to the value given by Theorell and Åkesson for purified cytochrome *c* (9). The high activity of this material was demonstrated by oxygen uptake in the presence of paraphenylenediamine and cytochrome oxidase in the Warburg respirometer.

A sample of cytochrome *c* prepared by this technique was diluted with water until 1 cc. gave an oxygen uptake of 250 to 300 c.mm. per hour in the presence of an excess of hydroquinone and cytochrome oxidase. Freshly prepared elementary bodies, in 5 to 15 mg. lots, were suspended in 1.5 cc. of phosphate buffer, mixed with 0.5 cc. of the diluted cytochrome *c* solution, and placed in a Warburg respiration flask. In the side arm was placed 0.5 cc. of water containing 3.6 mg. of hydroquinone. The flask was attached to the manometer, cock sealed, and shaken in the water bath at 37°C. When temperature equilibrium had been reached, the hydroquinone solution was tipped into the elementary body-cytochrome *c* mixture, and the manometer read at frequent intervals for oxygen uptake. Control flasks with cytochrome *c* and bydroquinone alone, were set up at the same time.

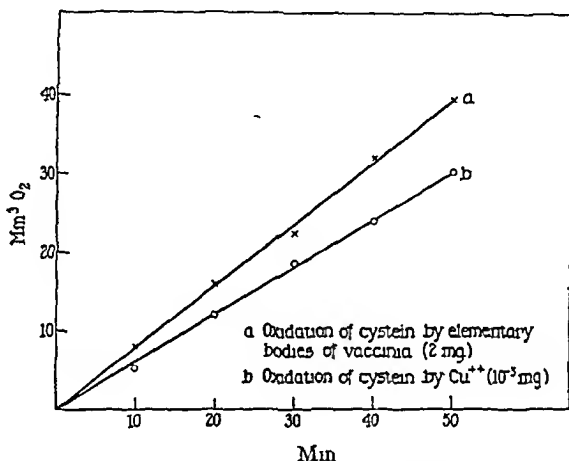
No significant oxygen uptake of the elementary body-cytochrome *c* hydroquinone mixture, over that of the control flasks, was observed after 2 hours when the experiment was discontinued. If the virus contained cytochrome oxidase it was not possible to demonstrate it by this technique.

Examination of Virus for Cytochrome c—A partially purified cytochrome oxidase for use in testing for cytochrome *c* was prepared from beef heart by the technique of Stotz and Hastings (10).

50 gm. of beef heart muscle were freed from fat and ligaments and ground twice in a meat chopper. The finely ground material was then placed in a bag made of two thicknesses of bandage gauze and washed in 1 liter of tap water at 40°C for 10 minutes, with occasional squeezing. This procedure was continued through 4 washings at 40°C, alternating with 4 washings at 15°C. The mass of washed material, squeezed dry, was next placed in a mortar and ground with 60 mesh alundum in 100 cc. of $M/15 K_2HPO_4$ until a smooth paste was obtained. The paste was allowed to stand at room temperature 30 minutes with occasional stirring. The material was then centrifuged at 2000 R.P.M. and the sediment discarded. To the supernatant, which was allowed to stand at 4°C overnight, was added an equal volume of 0.2 molar acetate buffer, pH 4.5. The mixture was then centrifuged and the supernatant

material discarded. The precipitate was resuspended in 10 cc of $M/15 K_2HPO_4$. 0.5 cc of this cytochrome oxidase preparation in the Warburg manometer was sufficient to yield an uptake of 20 to 30 c mm of oxygen per 10 minutes in the presence of an active preparation of cytochrome *c* and hydroquinone.

Purified elementary bodies, freshly prepared, in 5 to 15 mg lots were suspended in 1.5 cc of phosphate buffer and mixed with 0.5 cc of an active suspension of cytochrome oxidase in the Warburg respirometer flask. In the side arm was placed 0.5 cc of water containing 3.6 mg of hydroquinone. When temperature equilibrium had been achieved, the hydroquinone solution was tipped into the elementary body-cytochrome oxidase mixture, and the manometers read at frequent intervals for



TEXT-FIG 1 The oxidation of cysteine by purified elementary bodies of vaccinia oxygen uptake. A control, with cytochrome oxidase and hydroquinone alone, was set up at the same time.

No appreciable oxygen uptake of the elementary body-cytochrome oxidase-hydroquinone mixture, over that in the control flasks, was observed after 2 hours when the experiment was discontinued. When the experiment was repeated, paraphenylenediamine being used as a hydrogen donor, a significant oxygen uptake occurred which lasted over a period of 2 hours.

A much greater and more consistent oxygen uptake, however, was demonstrated when cysteine replaced paraphenylenediamine as a reducing agent. In this instance, the oxygen uptake was as great in the control system, which was composed of elementary bodies and cysteine, without the addition of cytochrome oxidase (Text-fig 1).

1 mg of fresh elementary bodies was suspended in 0.15 M buffer and placed in the bottom of a Warburg flask. In the side arm was placed 1 cc of an aqueous solution containing 6 mg of cysteine. Suitable controls, with elementary bodies and buffer

and cystein and buffer, were set up at the same time. After temperature equilibrium had been achieved, the cystein solution in the side arm was tilted into the respirometer flask, the manometers were read at 10 minute intervals for oxygen uptake. The rate of oxygen uptake from the oxidation of cystein by elementary bodies of vaccinia is shown in Text fig. 1

Identical rates of cystein oxidation by elementary bodies and by elementary bodies plus cytochrome oxidase indicated that no participation by cytochrome oxidase in this reaction was likely. With paraphenylenediamine, which is known from redox potential considerations to be less specific than hydroquinone in the enzymatic reduction of cytochrome *c*, the case is not so clear, since no appreciable oxidation of paraphenylenediamine by elementary bodies of vaccinia without added cytochrome oxidase could be demonstrated. Results obtained by the use of paraphenylenediamine are also rendered ambiguous by the fact that its oxidation is catalyzed by traces of cytochromes *a* and *b* which in most cases contaminate preparations of cytochrome oxidase.

Search for a Metallic Catalyst

The catalytic effect of metals on the oxidation of paraphenylenediamine (8) and cystein (11) is well known. That a metallic component was responsible for the catalysis noted above was further indicated by the effect of potassium cyanide which in a concentration of 0.002 molar was effective in completely blocking the reaction. Aa' dipyrldyl, which is known to prevent oxidative catalysis by traces of ferric ion, was wholly without effect, indicating that inorganic iron did not play a part in this reaction. Sodium diethyl-dithiocarbamate, however, was effective in completely preventing the oxidation of cystein by elementary bodies of vaccinia. This substance has long been known to block copper catalysis by irreversible combination with the copper ion, and, because of this reaction, can be made specific for the detection of copper if iron is previously bound by pyrophosphate or dipyrldyl (12).

The fact that the oxidation of cystein by elementary bodies of vaccinia was blocked effectively by sodium diethyl-dithiocarbamate made it likely that we were dealing with a copper constituent. It must be remembered that in higher concentration sodium diethyl-dithiocarbamate will block the catalytic effect of certain other metals, such as iron, cobalt, and magnesium. That the catalytic effect was due entirely to a metallic component was indicated by the fact that the asb from elementary bodies of vaccinia gave even a higher rate of cystein oxidation than would have been given by the intact virus.

Metallic ions often contaminate reagents, and our first thought was that in the preparation of elementary bodies of vaccinia copper had been introduced as a contaminant. Careful tests of the buffers and other reagents used in each step in the preparation of virus, however, failed to reveal a source of copper as a contaminant. Stainless steel gauze was substituted for bronze for the

scarification process which precedes the seeding of virus on the skin of rabbits without affecting the concentration of copper in the final product. Moreover, repeated washing of the purified virus resulted in no appreciable change in the concentration of the catalytic substance. Since in no instance could steps in the method of purification of the virus be shown to be responsible for the introduction of the relatively large amount of the catalytic substance observed in the purified virus, it was decided to attempt identification of the compound and find whether it was linked with virus activity.

Spectroscopic Demonstration of Copper in Vaccine Virus—For proof that the catalytic substance in the purified virus was copper, we turned to spectroscopic studies. Emission spectra procured for us by Dr. G. I. Lavin on several samples of purified virus revealed the lines of copper in each instance (Fig. 1).

The spectra were obtained by placing the dried materials in bored carbon electrodes which were then arced with 110 volts of direct current. The lower electrode, containing the test substance, was made the positive one in each case. The photographs were taken with a medium Hilger quartz spectrograph on 10 inch plates.

As a reference substance, 10 mg. of dried egg albumin, containing 0.05 per cent of added copper as copper sulfate, was likewise arced in a bored electrode. The resulting emission spectrum was compared with respect to position and intensity with that obtained from the purified virus. As a second reference substance 10 mg. of dried material separated from the virus in the last stage of purification (final horizontal sediment) was likewise arced, and the resulting emission spectrum was used for comparison.

When the emission spectrum of purified virus was photographed (Fig. 1a) the only lines to increase in intensity, over the residual traces given by the electrodes, were the copper lines at 3247 and 3274 Å, and those given by phosphorus in the region of 2530 to 2555 Å. The copper lines in the purified virus matched in position and intensity those given by the copper added to the egg albumin (Fig. 1b, d).

The spectrum obtained from the horizontal sediment (Fig. 1c) ordinarily discarded in the final stage of virus purification is interesting in that it contains a number of lines not seen in the purified virus. The major line at 3302 Å is thought to be due to zinc. Although this line and others due to metallic trace substances are absent from the final virus product, no diminution in intensity of the copper lines occurred. The absence of iron and other metallic constituents of tissue from the purified virus is highly significant in view of the original source of the virus, from animal skin. This is additional evidence that there is no appreciable quantity of impurities in our virus preparations.

Demonstration of Copper by Chemical Means—For quantitative determination of the copper constituent, several methods were available. The rate of cysteine oxidation in the absence of other metallic substances can in itself be made quantitative. Comparison of the rate of cysteine oxidation by elementary

bodies of vaccinia with the rate produced by known increments of copper ion revealed a copper content significantly over 0.03 per cent of the dry weight of the virus. For direct chemical determination we were able to employ successfully the method of Sachs *et al* (12), which could be performed on as little as 15 mg. of elementary bodies.

15 mg. of elementary bodies, dried to constant weight, were ashed at 600°C. in a vitreosil thimble with the aid of 0.25 cc. of reagent nitric acid. When ashing was complete, as revealed by the complete disappearance of carbon, the ash was extracted with 3 cc. of 6 N hydrochloric acid in 1 cc. amounts by warming the mixture to insure solution. The material was then transferred quantitatively to a 25 cc. volumetric cylinder, and followed by 2 additional rinses of the vitreosil thimble with 1 cc. of distilled water. A blank determination on reagents, including the ashing procedure, was run at the same time. 2 cc. of concentrated ammonia water was added and the solution cooled. 1 cc. of a 0.2 per cent aqueous solution of sodium diethyl-dithiocarbamate was next added and mixed thoroughly with the test solu-

TABLE I
Copper Content of Elementary Bodies of Vaccinia

Lot	Virus taken for analysis	Copper
	mg	per cent
1	15	0.051
2	15	0.056
3	15	0.052
4	15	0.048

tion by rotation of the tube. 10 cc. of isoamyl alcohol were next added, and the mixture shaken thoroughly for 1 minute. The yellow complex of copper and diethyl dithiocarbamate was extracted quantitatively by the isoamyl alcohol and appeared in the isoamyl alcohol layer which rose slowly to the top and formed a sharp interface with the aqueous layer below. The colored layer was removed by means of a pipette and transferred to a colorimeter cup and compared with a known copper standard treated in an identical manner. All reagents were tested for copper and made up in triple glass-distilled water. No test for copper could be obtained on the reagents alone.

The results of copper determinations made on four different lots of purified elementary bodies are given in Table I.

Nature of Copper Constituent

The fact that the copper constituent of our virus preparations was not appreciably altered by repeated washing of the purified virus indicated that the substance was held more firmly than by mere adsorption of inorganic copper ion.

Ultrafiltration of Elementary Bodies of Vaccinia—Ultrafiltration, in which the volume of wash water could be kept small, failed to lower significantly the

copper constituent, indicating that it was held firmly to the virus. Copper sulfate, as a source of copper ion, added in amounts 4 to 5 times that present in the virus, was readily removed in two washings.

10 mg of elementary bodies was suspended in 10 cc of distilled water and placed in a copper-free cellophane bag made of Visking sausage casing. The upper end was knotted, and the bag placed in an ultrafiltration tube similar to that described by Coolidge (13). The tube was then placed in a centrifuge and spun for 2 hours at 3500 R P M. After that time a measurable quantity of ultrafiltrate had appeared in the bottom of the tube below the glass constriction on which the cellophane tube rested. This ultrafiltrate was tested for copper by means of the cystein oxidation technique and resultant oxygen uptake in the Warburg respirometer. No significant trace of copper ion could be detected in the ultrafiltrate, and no diminution in copper concentration was detectable in the elementary bodies from which the ultrafiltrate had been removed. Added copper in amounts twice that already present in the virus, however, was readily removed by such treatment and could be recovered quantitatively in the ultrafiltrate.

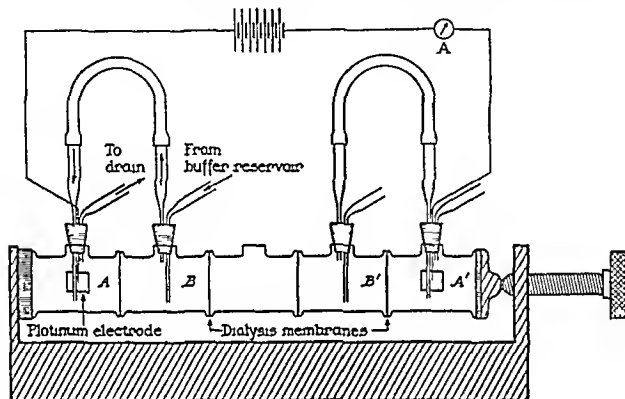
Electrodialysis of Elementary Bodies—Electrodialysis is one means of insuring removal from proteins of metallic substances which are not held in organic combination. The copper in certain of the purified copper proteins, such as tyrosinase and laccase, is not removed by this procedure. In our experience, the usual methods of electrodialysis render vaccine virus inactive. With ordinary electrodes this can be traced to rapid and uncontrollable pH changes due to electrolysis of buffer. Moreover, since the removal of metallic substances by electrodialysis is also affected greatly by small pH changes, it was necessary to devise some technique whereby pH could be carefully controlled and shifted easily by a variety of buffers. An electrodialysis unit, devised several years ago by Dr J H Bauer and Dr T P Hughes of the International Health Division of The Rockefeller Foundation, was modified according to the accompanying illustration and found quite satisfactory for our purposes.

5 glass cells, with ground contact joints, were separated by cellophane discs which were sealed to the contact joints by rubber cement. A platinum electrode was placed in each of the end cells and buffer of any desired molarity and pH, from a reservoir above, was allowed to run slowly into cells B and B' and over into A and A' by means of glass U-tubes, as shown in Text-fig 2. From the end cells, the buffer was allowed to drain away at a rate which was controlled by two screw clamps attached to the outlet tubes. The material to be dialyzed was placed in the middle cell, separated from the electrode cells by cells B and B' which were slowly but continuously rinsed with new buffer. pH determinations, done at frequent intervals with the glass electrode, revealed no changes in pH, either in the center or two adjacent cells, over a period of 3 to 4 days of continuous electrodialysis.

10 mg of freshly prepared elementary bodies of vaccinia were suspended in $\pi/20$

citrate NaOH buffer, and submitted to continuous electro dialysis for 36 hours at 20°C, with a potential of 110 volts and a current of 20 milliamperes. Samples of the suspension of virus were removed for copper analyses and infectivity studies at 6 hour intervals.

With M/20 citrate NaOH buffers, over a pH range of 6 to 8.5 no drop in copper concentration was observed. Moreover, no drop in infectivity of the virus over control samples of virus kept at the same temperature and pH was noted. Copper added to egg albumin in one instance and washed suspensions



TEXT FIG. 2 Modified Hughes Bauer electro dialysis apparatus in which the pH is controlled by means of circulating dilute buffer.

of *Lactobacillus casei* in another, in amounts equivalent to that found in vaccine virus, was readily and completely removed within 4 to 6 hours over the same pH range. Finally, copper ion added to virus suspensions could likewise be removed by this technique.

Concentration of Copper Constituent with Purification of Virus—A study of the virus material at various stages of purification has yielded certain information concerning the constituents which make up the purified virus. The virus constituents are necessarily concentrated as the purification proceeds, while the constituents representing contaminants arising from the skin of the rabbit tend to disappear (1). A study of the virus material during the process of purification showed a striking increase in the amount of copper.

A portion of the dermal pulp scraped from the skin of rabbits infected with vaccinia was separated from the virus by differential centrifugation as described in an

copper constituent, indicating that it was held firmly to the virus. Copper sulfate, as a source of copper ion, added in amounts 4 to 5 times that present in the virus, was readily removed in two washings.

10 mg of elementary bodies was suspended in 10 cc of distilled water and placed in a copper-free cellophane bag made of Visking sausage casing. The upper end was knotted, and the bag placed in an ultrafiltration tube similar to that described by Coolidge (13). The tube was then placed in a centrifuge and spun for 2 hours at 3500 R P M. After that time a measurable quantity of ultrafiltrate had appeared in the bottom of the tube below the glass constriction on which the cellophane tube rested. This ultrafiltrate was tested for copper by means of the cysteine oxidation technique and resultant oxygen uptake in the Warburg respirometer. No significant trace of copper ion could be detected in the ultrafiltrate, and no diminution in copper concentration was detectable in the elementary bodies from which the ultrafiltrate had been removed. Added copper in amounts twice that already present in the virus, however, was readily removed by such treatment and could be recovered quantitatively in the ultrafiltrate.

Electrodialysis of Elementary Bodies—Electrodialysis is one means of insuring removal from proteins of metallic substances which are not held in organic combination. The copper in certain of the purified copper proteins, such as tyrosinase and laccase, is not removed by this procedure. In our experience, the usual methods of electrodialysis render vaccine virus inactive. With ordinary electrodes this can be traced to rapid and uncontrollable pH changes due to electrolysis of buffer. Moreover, since the removal of metallic substances by electrodialysis is also affected greatly by small pH changes, it was necessary to devise some technique whereby pH could be carefully controlled and shifted easily by a variety of buffers. An electrodialysis unit, devised several years ago by Dr J H Bauer and Dr T P Hughes of the International Health Division of The Rockefeller Foundation, was modified according to the accompanying illustration and found quite satisfactory for our purposes.

5 glass cells, with ground contact joints, were separated by cellophane discs which were sealed to the contact joints by rubber cement. A platinum electrode was placed in each of the end cells and buffer of any desired molarity and pH, from a reservoir above, was allowed to run slowly into cells B and B' and over into A and A' by means of glass U-tubes, as shown in Text-fig 2. From the end cells, the buffer was allowed to drain away at a rate which was controlled by two screw clamps attached to the outlet tubes. The material to be dialyzed was placed in the middle cell, separated from the electrode cells by cells B and B' which were slowly but continuously rinsed with new buffer. pH determinations, done at frequent intervals with the glass electrode, revealed no changes in pH, either in the center or two adjacent cells, over a period of 3 to 4 days of continuous electrodialysis.

10 mg of freshly prepared elementary bodies of vaccinia were suspended in M/20

time, but against phosphate buffer alone. The reduction in titre in both instances may have been due to agglutination occurring as a result of increased electrolyte concentration.

Studies of Enzymatic Activity of the Copper Constituent—A great many copper proteins are now known (5). Hemocyanin, hemocuprein, hepatocuprein, polyphenol oxidase, laccase, and tyrosinase are representatives of a group of proteins containing copper in organic combination. The three latter substances are enzymes and are active in the oxidative catalysis of certain benzenoid compounds. On the basis of the possibility that a similar enzymatic rôle could be ascribed to the copper constituent in the elementary body of vaccinia, the effect of the intact virus on a number of substrates of the benzenoid series was studied.

1.5 cc. of $M/150$ catechol in phosphate buffer, pH 7.7, were placed in the side arm of a Warburg respiration flask. 2 mg. of freshly prepared elementary bodies of vaccinia suspended in phosphate buffer were placed at the bottom of the vessel. Controls of substrate and buffer without virus were set up at the same time. The manometer cocks were closed and the shaking apparatus set at 80 per minute. When temperature equilibrium had been achieved, the catechol substrate was tilted into the elementary body suspension and the manometers observed at 10 minute intervals for oxygen uptake. No reaction was observed in 2 hours when the experiment was discontinued.

A similar attempt to demonstrate the catalytic oxidation of other benzenoid compounds was made. No oxygen uptake was detected when paraphenylenediamine, hydroquinone, orcinol, phloroglucinol, benzidine, or tyrosine was used as a substrate with 2.0 mg. of freshly prepared virus in a system similar to that described in detail for catechol.

DISCUSSION

It is interesting that a certain degree of parallelism between virus purification and copper concentration can be demonstrated. This is particularly significant in view of the fact that other metallic constituents present in the early stages of virus purification are absent from the final product in so far as can be demonstrated spectroscopically. The fact that no significant spectroscopic traces of iron compounds exist in the purified virus indicates, we believe, that no appreciable contamination of the purified virus with cellular debris is likely.

The demonstration of a copper constituent associated with the purified elementary body of vaccinia further emphasizes the complexity of the elementary body of vaccinia. That the material is not one of the known copper protein enzymes is fairly certain, as evidenced by its lack of reactivity with the known substrates of these substances. Failure to be removed by electro dialysis over a pH range in which the virus remains active indicates that the

earlier publication dealing with the purification of elementary bodies of vaccinia (1). This material was washed several times in dilute buffer and finally sedimented by prolonged centrifugation and dried from the frozen state. Successive sediments, ordinarily thrown away in the course of virus purification, were likewise retained, washed in successive changes of dilute buffer, dried from the frozen state, and analyzed for copper. The values for copper obtained after ashing of the dried sediments are recorded in Table II, together with the copper content of the purified virus separated from these successive sediments.

The successive analyses (Table II) show that a 25-fold increase in copper concentration occurred during the process of purification of elementary bodies of vaccinia. The relatively high copper content of the final sediment, which is ordinarily discarded during the process of virus concentration and purifica-

TABLE II

A Comparison of the Copper Content of Vaccine Virus with That of Materials Discarded during Purification of the Virus

Material	Material taken for analysis	Copper
	mg	per cent
Crude dermal pulp	20	0.002
"First horizontal sediment"	20	0.005
"Final horizontal sediment"	20	0.040
Purified virus	20	0.050

tion, is not surprising since it is known to contain large amounts of the active agent.

Dialysis of Elementary Bodies against Potassium Cyanide Solution—Kubowitz (14) has claimed to show that the copper constituent of certain copper protein enzymes can be removed by dialysis against a dilute solution of potassium cyanide, with resultant loss of activity of the protein. Upon addition of copper in the form of inorganic salts, the activity of the protein is restored. An attempt to remove the copper from vaccine virus by dialysis against potassium cyanide was unsuccessful.

20 mg of freshly prepared elementary bodies of vaccinia were suspended in dilute phosphate buffer in a cellophane sac and dialyzed with frequent agitation against 0.1 molar potassium cyanide at pH 7.2 for 48 hours at 4°C. The virus was next dialyzed against distilled water until free of cyanide, after which the virus suspension was frozen and dried to constant weight *in vacuo*.

An analysis of this preparation by means of ashing and color produced with sodium diethyl-dithiocarbamate revealed no significant loss of copper. The infectivity of this material, although dropping somewhat, was not less than that observed in a comparable virus control dialyzed for the same period of

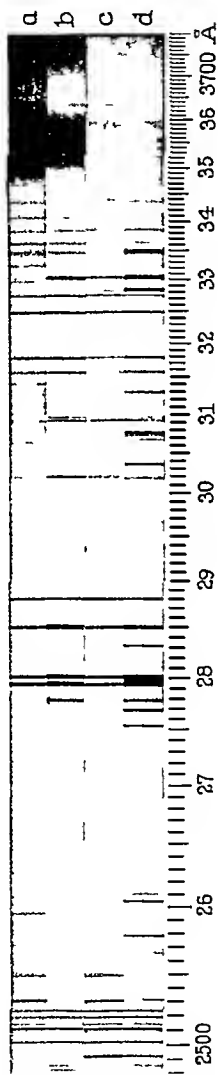


FIG 1

copper is bound with a degree of firmness exhibited by known copper proteins, such as tyrosinase and hemocyanin. It is tempting to assume that a metallic group such as copper, by virtue of its known rôle as an oxidative catalyst, may possibly function in the respiratory activity of the virus. No proof of such a function can be furnished at this time.

SUMMARY

A search by means of spectroscopic and enzymatic techniques has failed to demonstrate either cytochrome or cytochrome oxidase in purified elementary bodies of vaccinia. A constituent of the virus which catalyzes the oxidation of cystein has been found and identified as copper in a concentration amounting to 0.05 per cent of the dry weight of the virus. The copper constituent was not removed by repeated washing, ultrafiltration, dialysis against 0.1 molar potassium cyanide, or by electrodialysis over a pH range which did not inactivate the virus. During the process of purification of the virus a 25-fold increase of the copper constituent was observed. Emission spectra obtained from the dry virus also revealed copper but no significant traces of other metallic substances. No biological rôle can yet be ascribed to the copper component of virus.

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EXPLANATION OF PLATE 6

FIG. 1. Emission spectra of purified elementary bodies of vaccinia (a), of egg albumin to which copper was added to 0.05 per cent concentration (b, d), and of material removed from elementary bodies of vaccinia in final stage of purification (c).

THE EFFECT OF THE VIRUS HOST CELL RELATIONSHIP ON INFECTION WITH VACCINIA*

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(Received for publication, April 12, 1941)

In the course of certain recent experiments (1) it was observed that the chance of a virus preparation causing infection in an animal could not be predicted solely by the amount of virus injected and by the host's resistance but was influenced by still another variable. It seemed possible that this variable might be the tissue mass (number of cells) exposed to each virus particle. If this were true it would be a new aspect of the well recognized obligate parasitism of viruses and would be also of practical importance in experiments concerned with titration of viruses in animals. In order to examine this possibility experiments were designed in which the amount of virus and the host resistance were kept constant but the number of host cells exposed to the virus varied. This was done by three types of experiments (1) varying the amount of inoculum, (2) localizing and (3) increasing the spread of a constant quantity of virus. Localization was accomplished by injecting the animals with estrogenic hormone which Sprunt and McDearman (2) had shown to localize the spread of particulate material. Increase in spread was accomplished by adding an aqueous extract of testis to the virus preparation following the work of Duran Reynals (3). These experiments all showed that the tissue mass (number of cells) exposed to the virus was an important factor in predicting the chance of a lesion.

Methods and Materials

Animals—Normal, white, adult male rabbits weighing about 2 kilos were used.

Virus—The virus employed was a strain of vaccine lymph obtained from the North Carolina Laboratory of Hygiene, Raleigh. This strain has been described in detail (4). This virus had been passed serially more than 50 times in rabbit skin. It was purified as described by Craigie and Wishart (5) and Parker and Rivers (6). Measured amounts of this virus were rapidly lyophilized and kept sealed in a vacuum until ready for use. This virus preparation is referred to in this paper as 'ordinary' virus to distinguish it from the other preparation described below.

* Reported in abstract before the American Society for Experimental Pathology, April 16, 1941. Aided in part by a grant from the Duke University Research Council.

(From the

In the course of a virus preparation solely by the amount influenced by stimulation might be the tissue this were true in the case of virus resistance concerned with the possibility of host resistance the virus varied the amount of constant quantity of animals with estrogen to localize the spread by adding the work of tissue mass (number) in predicting the

Animals—None
Virus—The virus was prepared at the Carolina Laboratory (4) This virus was purified as described in amounts of this ready for use. To distinguish it from

* Reported in April 16, 1941

the number of virus particles in the inoculum. For example, in Experiment 1 it is seen that when 0.50 cc. of the various virus dilutions was used a 50 per cent point of 7.08 (1:12,000,000) resulted. But when a 0.10 cc. inoculum was employed, that is, 1/5 the number of virus particles were injected, the 50 per cent point obtained was 7.04 (1:11,000,000) instead of 6.39 (1:2,400,000). These results indicate that the smaller inoculum was relatively the more ef-

TABLE I
Effect of Size of Inoculum on 50 Per Cent Point

Volume of inoculum cc.	No. of injections of each dilution	Log of 50 per cent point
Experiment 1		
0.50	24	7.08
0.10	24	7.04
Experiment 2		
0.50	16	7.89
0.10	16	7.83
0.05	16	7.72
Experiment 3		
1.00	20	8.13
0.25	24	7.96
0.05	24	7.66
Experiment 4		
2.00	18	8.41
0.25	24	8.41
0.05	24	7.92
Experiment 5		
1.00	24	8.48
0.25	24	8.37
0.05	24	7.91

fective. Similar results are seen in the other experiments. The explanation of this apparent paradox is that the number of cells exposed to each virus particle in the smaller inoculums was greater than the number exposed to each particle in the larger inoculums. Although the concentration of the particles in 1 cc. and 0.25 cc. was the same, exposing when injected the same number of cells per particle, this equality was destroyed following injection because of the conditions under which spread must take place in the skin. Proof of this point is based on studies of various sized inoculations of India ink suspensions.

The method of making the injections of ink and measuring their spread is given elsewhere (2). When a solution is injected in the skin it takes the form of a disc

The strain of virus which for convenience in this paper we have called the "estrogenic passage strain" was prepared as follows. The "ordinary" virus was passed serially through 10 rabbits each of which had been injected with the estrogenic hormone for 3 weeks. After the 10th passage the virus was purified and sealed in a vacuum as described above.

Hormones—The estrogenic hormone used was estradiol dipropionate,¹ each dose of which contained 0.2 mg. in 0.25 cc. of sesame oil. Single daily injections were made for 3 weeks prior to injection of the virus and in each case were continued until the end of the experiment. The control rabbits were given 0.25 cc. of sesame oil daily but no hormones.

Spreading Factor—Adult bull testicular tissue was triturated with alundum, 1 cc. of Locke's solution was added for each gram of testis, the suspension was centrifuged, and the supernatant fluid was stored in the refrigerator. Just prior to use, this stock solution was diluted 1:33 with Locke's solution and then added in equal parts to the virus dilutions making a final dilution of 1:66. A 0.10 cc. inoculation of the testicular extract when mixed with India ink in this dilution was found to have a mean spread in 4 hours of 6.33 sq. cm.

Titration—Since the approximate strength of the virus was known, 7 twofold dilutions could be made so that inoculums from the most dilute one would cause either no lesions or only a few and inoculums from the least dilute would cause lesions in every instance. Only 1 set of dilutions was made for each experiment. In the *Variation in Size of Inoculum Experiments*, different sized samples of the virus dilutions were used. In the *Localization of Spread Experiments* the same dilutions in 0.25 cc. amounts were inoculated into both the control and the treated animals. In the *Increased Spread Experiment* half of each dilution was mixed with a 1:33 suspension of the aqueous testicular extract. Then 0.10 cc. of each dilution of the virus without the testicular extract was injected into each rabbit in 1 group and the same amount of the virus with the spreading factor into each rabbit of another group. Separate groups of rabbits had to be used in this experiment, as the virus with spreading factor caused the lesion to appear much sooner than did the control virus and frequently to spread over the control lesions. In all except the first experiment in this group decimal instead of twofold dilutions were used.

EXPERIMENTAL

Variation in Size of Inoculum Experiments—These experiments were designed to determine the relationship between the volume of the inoculum and the probability of a lesion. Table I shows the results obtained. Column 2 of this table shows the number of each of 7 twofold dilutions injected. Column 3 shows the logarithm of the 50 per cent point. The 50 per cent point is that dilution of the virus preparation from which 50 per cent of the inoculums, when injected intradermally, produce positive lesions. All of these experiments show that the 50 per cent point does not vary in direct proportion to

¹ The alpha-estradiol dipropionate (di-ovocylin) was kindly supplied by the Ciba Pharmaceutical Products, Inc.

Localization of Virus Experiments—In previous publications (2, 7) it was shown that the estrogenic hormone localized particulate matter in the skin and also increased the resistance of the rabbit to infection with vaccinia. It was thought that this increased resistance was due either to the localization of the virus or to the fact that the estrogenic hormone had so changed the treated animals as to render them less susceptible. The latter was considered likely as it is well known that the serial passage of a virus through one type of host increases the virulence of the virus for this host. If the estrogenic hormone had changed the host and if the virus was adapted by serial passage to animals which had received this hormone, then the animals receiving this hormone should be more susceptible to the "estrogenic passage virus" than the untreated rabbits. This experiment was designed, therefore, to determine which of these factors was the important one. The results are shown in Table II. It is seen in this table that a larger amount of both the "ordinary" and "estrogenic

TABLE III
Effect of Dispersion of Inoculum on 50 Per Cent Point

	Control		Spreading factor	
	No. of injections of each dilution	Log of 50 per cent point	No. of injections of each dilution	Log of 50 per cent point
Experiment 1	18	6.3	18	7.5
Experiment 2	12	5.5	6	6.6
Experiment 3	12	5.6	6	6.4
Experiment 4	12	5.4	6	6.3

passage virus" was required to infect the animals injected with the estrogenic hormone than was required to infect the controls. Hence of these two possibilities the localization of the virus is the important one.

Increased Spread Experiments—Duran Reynals (3) has shown clearly that the addition of an aqueous testicular extract to vaccinia increases the size of the lesion and also increases the spread of India ink. Reasoning from these facts, it would seem that use of the spreading factor would expose a greater number of cells to each virus particle. The purpose of this experiment was to show whether, under such circumstances, the probability of a lesion would be increased. Table III shows the results obtained. It is seen that when the virus is mixed with the spreading factor it takes only about 1/10 as much virus to infect as it does without the spreading factor. When the latter is used, the lesions are difficult to read as they are quite diffuse. Wherever there was any question regarding the presence of a lesion we have in every instance considered it as no lesion, hence it is thought likely that the results obtained should have been greater thus making a larger divergence between the virus with and without the spreading factor.

with a slight bulge at point of injection. Since the skin is of uniform thickness a measurement of the surface area covered by the India ink gives a reasonably precise measurement of the volume of tissue involved. The surface area covered by 1 cc was found immediately after injection to be 4.71 sq cm and an inoculum of 0.25 cc was found to spread over 1.20 sq cm. These areas manifestly have the same relationship to each other as did the volumes of the inoculums. After 1 hour the spread of 1 cc of India ink was 8.31 sq cm and 0.25 cc was 3.88 sq cm. This approximate 2

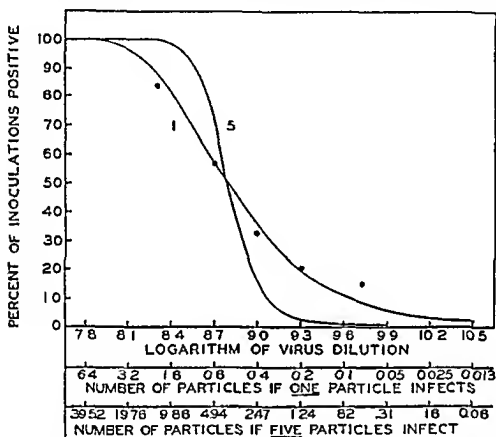
TABLE II
Effect of Localization of Inoculum on 50 Per Cent Point

	No. of injections of each dilution	Log of 50 per cent point	Mean spread of India ink in 4 hrs
Experiment 1			sq cm
Ordinary virus			
Control animals	28	6.54	11.45
Estrogen animals	23	6.20	9.04
Difference		0.34	2.41
Estrogenic passage virus			
Control animals	27	6.29	11.45
Estrogen animals	24	5.92	9.04
Difference		0.37	2.41
Experiment 2			
Ordinary virus			
Control animals	28	6.01	10.53
Estrogen animals	28	5.77	8.53
Difference		0.24	2.00
Estrogenic passage virus			
Control animals	28	5.90	10.53
Estrogen animals	28	5.61	8.53
Difference		0.29	2.00

to 1 ratio had remained constant when measurements were made at 2 and 4 hours. It is apparent therefore, that twice as many cells were exposed to each virus particle in the 0.25 cc inoculum as were exposed to each particle in the 1 cc inoculum. For example, if the 1 cc inoculum involved 80 cells then 0.25 cc would involve 40 cells. If the dilution injected contains 8 virus particles per cc, then with 1 cc inoculum we would have 1 virus particle per 10 cells and in 0.25 cc inoculum 1 virus particle per 20 cells.

That the ratio should change from 4 to 1 to 2 to 1 following inoculation is understandable when it is realized that the injected material takes the form of a disc in the skin and can only spread from the periphery of this disc. The circumference of the disc from 1 cc (4.71 sq cm) and 0.25 cc (1.20 sq cm) are found to be 7.73 cm and 3.88 cm which have a 2 to 1 ratio.

samples which would contain 1 particle or 2 particles or n particles and to draw series of curves using the number of particles as the abscissa and the percentage positive as the ordinate. Or, if we know the percentage of inoculations positive for any given dilution it is then possible to predict the number that should be positive for any other dilution as shown in Text fig 1. The slope of the curve would become steeper in proportion to the increase in the number of particles necessary to infect in any given sample. In actual practice the procedure is to take some point, generally the point indicating that 50 per cent of the inoculations are positive and 50 per cent negative, and construct the curves for 1 or more particles from this point. The curve which the



TEXT FIG 1 Experimental data obtained with a preparation of vaccinia virus. The curve with the steeper slope is 0.5 particle curve. The other a 1 particle curve. The dots represent the experimental data.

experimental data come closest to fitting is considered the correct one. Then the number of particles present in any dilution can be read off.

Parker (8) showed that vaccinia followed the 1 particle curve more closely than any of the other curves and later found a similar result with infectious myxoma (11). Sprunt and McDearman (7) verified Parker's finding with respect to a fit with the 1 particle curve but thought the results indicated that 1 particle would infect if it reached a susceptible cell. The possibility of a change in cell susceptibility accounting for a part of the results reported in the above experiments is not to be lost sight of. However, even if cell susceptibility is changed, an increase or decrease in the total number of cells

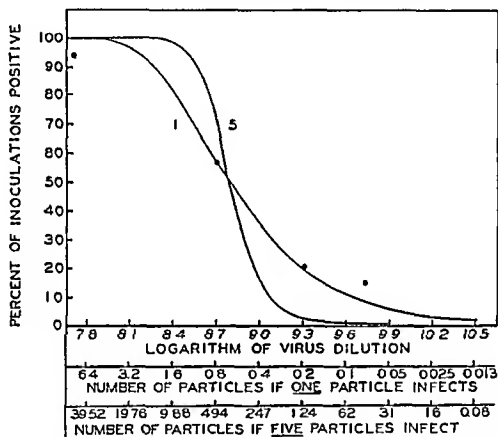
DISCUSSION

These experiments show clearly that the chance of a virus preparation causing a lesion in an animal is dependent upon the volume of tissue exposed as well as upon the amount of the virus injected and the resistance of the host. In other words the more cells per virus particle the greater the chance of a lesion. This fact has been shown by three types of experiments (1) varying the volume of inoculum resulted in more cells being exposed per virus particle in smaller inoculums than in the larger, and hence in a larger number of infections with the smaller ones than was predicted on the basis of the volume of the inoculum alone, (2) localizing the virus with the estrogenic hormone exposed to it a smaller number of cells and reduced the incidence of infection, (3) spreading the virus over a greater volume of tissue increased the incidence of infection.

The fact that the tissue mass exposed to a virus influences the result, like the amount of virus and the host resistance, reiterates the importance of the host cell in virus experimentation. It also introduces a variable which increases the possibility of error in attempting to obtain absolute data concerning the number of virus particles present by animal titration. It places, moreover, especial difficulty in the path of applying the Poisson law of small numbers to virus work. The use of this law in virus experimentation was described by Parker (8) in 1938. Its employment however is fraught with difficulty as it depends on a close correlation between the number of virus particles and the host response curve. Some of these difficulties have been previously discussed by Bryan and Beard (9) and by Sprunt, Marx, and Beard (10). The fact brought out in this paper that the ratio of virus particles to cells is also a variable proves also that the correlation between the number of particles and the host response cannot be perfect and that the goodness of fit of experimental data to a 1 or more particle Poisson curve could have no significance.

To make this point clear it seems advisable to review briefly the application of the Poisson law of small numbers to virus experimentation. The following procedures constitute this method. First, the approximate strength of the virus is determined by decimal titration. Second, animals are inoculated with a number of samples from each of 5 or more twofold dilutions of the virus preparations, these dilutions being so chosen that the highest one will give only a few positive lesions and the lowest one approximately 100 per cent lesions. Third, the results of these inoculations are plotted, indicating on the ordinate the percentage positive for each dilution and, on the abscissa, the viral dilutions. (See Text-fig 1). Fourth, the Poisson law of small numbers is applied to these data. This law states that if successive samples are drawn from a universe which consists of very small particles suspended in a liquid, the proportion of samples which contains 1 or more particles is definitely related to the mean number of particles per unit volume. Thus it is possible, if we know the number of particles present in a series of dilutions, to calculate the percentage of a number of

samples which would contain 1 particle or 2 particles or n particles and to draw series of curves using the number of particles as the abscissa and the percentage positive as the ordinate. Or, if we know the percentage of inoculations positive for any given dilution it is then possible to predict the number that should be positive for any other dilution as shown in Text fig 1. The slope of the curve would become steeper in proportion to the increase in the number of particles necessary to infect in any given sample. In actual practice the procedure is to take some point, generally the point indicating that 50 per cent of the inoculations are positive and 50 per cent negative, and construct the curves for 1 or more particles from this point. The curve which the



TEXT FIG 1 Experimental data obtained with a preparation of vaccinia virus. The curve with the steeper slope is a 5 particle curve. The other a 1 particle curve. The dots represent the experimental data.

experimental data come closest to fitting is considered the correct one. Then the number of particles present in any dilution can be read off.

Parker (8) showed that vaccinia followed the 1 particle curve more closely than any of the other curves and later found a similar result with infectious myxoma (11). Sprunt and McDearman (7) verified Parker's finding with respect to a fit with the 1 particle curve but thought the results indicated that 1 particle would infect if it reached a susceptible cell. The possibility of a change in cell susceptibility accounting for a part of the results reported in the above experiments is not to be lost sight of. However, even if cell susceptibility is changed, an increase or decrease in the total number of cells

exposed will also increase or decrease the number of susceptible cells exposed. That a change in the host cell virus particle ratio without any change in cell susceptibility will produce the changes reported above is clearly shown by another fact mentioned by Sprunt and McDearman (7). They called attention to the fact that the slope of the curve drawn through the experimental points was always less than the slope of the 1 particle curve. This is true not only in their experiments but also holds good for Parker's (8, 11) with vaccinia and infectious myxoma and for Bryan and Beard's (9) with papilloma. Sprunt and McDearman (7) suggested that this uniform deviation from the slope of the 1 particle curve might be significant and the results of the experiments reported in this paper make possible an explanation. This explanation can best be shown by an example. If one has 7 twofold dilutions of a virus preparation and these dilutions contain 8, 4, 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$ virus particles for each inoculum used, and the inoculum exposed 2048 cells to the virus then the ratio of virus particles to number of cells would range from 1 virus particle per 256 cells in the lowest dilution to 1 virus particle per 2048 cells in the highest dilution. As it is shown in this paper that the probability of a lesion is increased by increasing the number of cells exposed to each virus particle, in any virus titration in animals the number of cells per virus particle increases as the dilutions become weaker and the probability of a lesion also increases. This would result in the higher dilutions being more effective than the Poisson law of small numbers would indicate and the lower dilutions less effective. Since the Poisson curve is constructed from the 50 per cent point, the slope of a curve through the experimental point would not be as steep as the 1 particle curve. This is seen to be true of the data shown in Text-fig 1. Obviously there will always be a deviation from the Poisson curve, so that in any actual experiment we would not know whether this deviation was a 1 particle Poisson curve or a 100 particle curve. Hence, a fit to a Poisson curve can have no significance in regard to number of virus particles present in the preparation.

CONCLUSIONS

Experiments are described in which it is shown that, in addition to the amount of virus injected, the chance of a lesion also depends on the tissue mass (number of cells) exposed to the virus shortly after injection, and that the larger the number of host cells per virus particle the greater the probability of a lesion. This point has been shown by three types of experiments: (1) varying the size of the inoculum showed that the smaller sizes were relatively more effective than the larger ones, (2) localizing the virus by the estrogenic hormone decreased the chance of a lesion occurring, (3) spreading the virus over a larger area increased the probability of a lesion.

It is also shown that because the ratio of virus particles to host cells varies and because this ratio partly determines whether a lesion occurs, the number of

particles of virus cannot be predicted by the use of the Poisson law of small numbers

Since this paper was sent to press a preliminary publication by Olitsky and Schlesinger (12) has appeared which corroborates the conclusions of these experiments. These authors show that the inoculation of herpes virus into the skin of mice previously prepared by the injection of hypertonic saline increases the susceptibility of the mice to infection with this virus. They advance the hypothesis that this increased susceptibility is due to the fact that an increased number of nerve fibers are exposed to the virus.

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THE CARDIAC FACTOR IN THE "PRESSOR" EFFECTS OF RENIN AND ANGIOTONIN

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Since Tigerstedt and Bergman (1), in 1898, described the pressor effects of renal extracts, and to the active principle applied the name "renin," the vasoconstrictor action of this and similar substances has received particular attention and emphasis. This effect is, indeed, the basis of certain standard tests for the presence and potency of "renal pressor substances" in blood or tissue extracts (2-7). Moreover, as applied to these and other blood pressure-raising substances, the term "pressor" has come to be loosely used to connote, or to imply, vasoconstriction. The control of blood pressure rests, however, upon the coordinate interplay of several factors, of which peripheral vascular resistance is but one, other factors are cardiac output and volume of circulating blood.

The authors have examined the influence of renin and angiotonin upon various segments of the circulation (8, 9) and report herewith the effects of these substances upon the heart and coronary circulation.

Tigerstedt and Bergman (1) observed no consistent variation of pulse rate following intravenous injection of renin, nor any effect of renin upon the isolated, perfused heart of the rabbit. Hessel (10) confirmed these results using the perfused hearts of frogs and the isolated auricles of guinea pigs which were uninfluenced even by huge doses of renin. He further reported that in concentrations below 1:20,000, renin was without effect upon isolated strips of the coronary arteries of cattle. In a tabular summary (11) comparing the actions of adrenalin, renin, and 'vasopressin' Hessel stated the action of renin upon the heart to be *Förderung nur bei geschädigtem Herzen*."

Effects upon the Isolated, Perfused Heart

Renin was prepared by alcohol precipitation of fresh pig's kidney cortex, and fractional precipitation with ammonium sulfate, followed by prolonged dialysis. Renin activator was prepared from beef serum by fractional precipitation with ammonium sulfate, dialysis, and concentration by evaporation at room temperature. Angiotonin

* Working under the Jacques Loeb and Archbold Fellowships

was prepared by the method of Page¹ and Helmer (12) The pH of this solution was adjusted to 7.0 with dilute sodium hydroxide just prior to injection

The hearts of cats were isolated and perfused with Ringer-Locke solution by the Langendorff method The solution to be tested was slowly injected, in doses shown to produce minimal to moderate pressor effects in intact animals, into the stream of the perfusate just above the heart The rate of outflow from the coronary arteries was measured and recorded by means of a Condon (13) magnet-tipper A myographic tracing of the contractions of the right ventricle was registered on a kymograph In several experiments electrocardiographic records were obtained by means of worsted electrodes attached to the heart over base and apex

Results

Renin—The observations that renin is without significant influence upon the isolated heart, perfused with Ringer-Locke solution, were entirely confirmed by 33 injections in 16 experiments Occasionally coronary outflow was slightly reduced for a short interval, but rate or amplitude of beat was never affected (See Table I)

Renin Activator—This substance was tested in only four experiments Aside from an evanescent reduction of coronary flow, due perhaps to its protein content, renin activator was also without influence upon this preparation (See Table I)

Angiotonin—

Coronary Flow—Twenty-four injections in 12 experiments reduced the coronary outflow from 27 to 86 per cent (average 45 per cent) in the presence of normal sinus rhythm Representative results in seven experiments are summarized in Table I The effect reached its maximum within 30 to 75 seconds after the beginning of the injection and lasted for 1 to 6 minutes (See Fig. 1) This showed no tendency to diminish with successive injections, though not more than four were administered to any one preparation One or more previous exposures to renin did not appear to influence the reaction to subsequent injections of angiotonin In one experiment a cat weighing 2.8 kilos was rendered tachyphylactic to renin by five injections of from 2 to 5 cc The pressor response to angiotonin was then considerably reduced by ten successive injections of from 1 to 5 cc Following these procedures the heart was removed and perfused In this preparation (E-31) angiotonin (0.9 cc of the same batch administered to the animal *in vivo*) caused a striking reduction in coronary outflow

A moderate rise in coronary flow (averaging 16 per cent for all injections) frequently followed the early reduction caused by angiotonin This late increase in flow was measurably reduced with successive injections

Such diminution of coronary flow is not, apparently, secondary to any effect

¹ The authors are indebted to Dr. Page who kindly furnished us a quantity of angiotonin, this was uniformly more concentrated than our own preparation

of angiotonin upon speed or amplitude of ventricular contraction. Four injections, in three preparations in which ventricular fibrillation was maintained

TABLE I

Effect of Angiotonin, Renin and Renin Activator upon the Isolated Cat's Heart

Experiment No.	Substance tested	Coronary flow			Amplitude of beat			Heart rate	
		Initial flow	Maximal reduction	Duration reduced flow	Initial amplitude	Maximum change	Duration change	Initial rate	Change of rate
	cc	cc/min	per cent	min	mm	per cent	min	beats/min	per cent
E 23	A 0.6	50	56	2	22	+91	6	140	-1
	A 0.6	45	33	2	21	+72	4	132	-9
	A 0.7	36	59	3	24	+63	7	116	-8
E 24	A 0.6	38	53	1	22	+250	6	124	-1
	R 0.5	30	3	1	21	+4	2	105	-1
	A 0.5	21	42	2	22	+50	6+	108	-8
E 25	A 0.45	34	69	2	17	+200	9	115	-8
	A 0.45	34	47	2	19	+35	6	120	-8
	A 0.45	23	50	3					
E 26	RA 2.5	41	20	1	15	0		106	-4
	R 2.0	45	10	1	14	0		102	0
	A 0.5	41	56	2	15	+250	12	108	+9
	R 2.0	34	0		15	0		120	0
	**RA 2.5	36	0		18	-10		120	0
E 27	RA 2.0	36	+20	1½	23	0		115	0
	R 2.0	38	13	2	22	0		115	0
	A 0.3	38	29	2	21	+72	6	115	+4
	RA 2.0	34	0		18	0		120	-4
E 29	A 0.6	67	30	2	16	+260	7+	144	-2
	*A 0.15	54	30	3					
E 31	A 0.9	63	67	3	7	+300	5	123	-11

R renin A angiotonin RA renin activator

* Ventricular fibrillation maintained throughout observation by faradic stimulation of ventricles

** In this observation activator was boiled and supernatant fluid injected

by faradic stimulation brought about a decrease in coronary flow by from 16 to 48 per cent (average 28 per cent)

Heart Rate—Significant effects upon heart rate were not recorded. In some observations the rate of beat diminished slightly during the period of reduced coronary flow. In a few others there was an evanescent slight acceleration at the height of the effect.

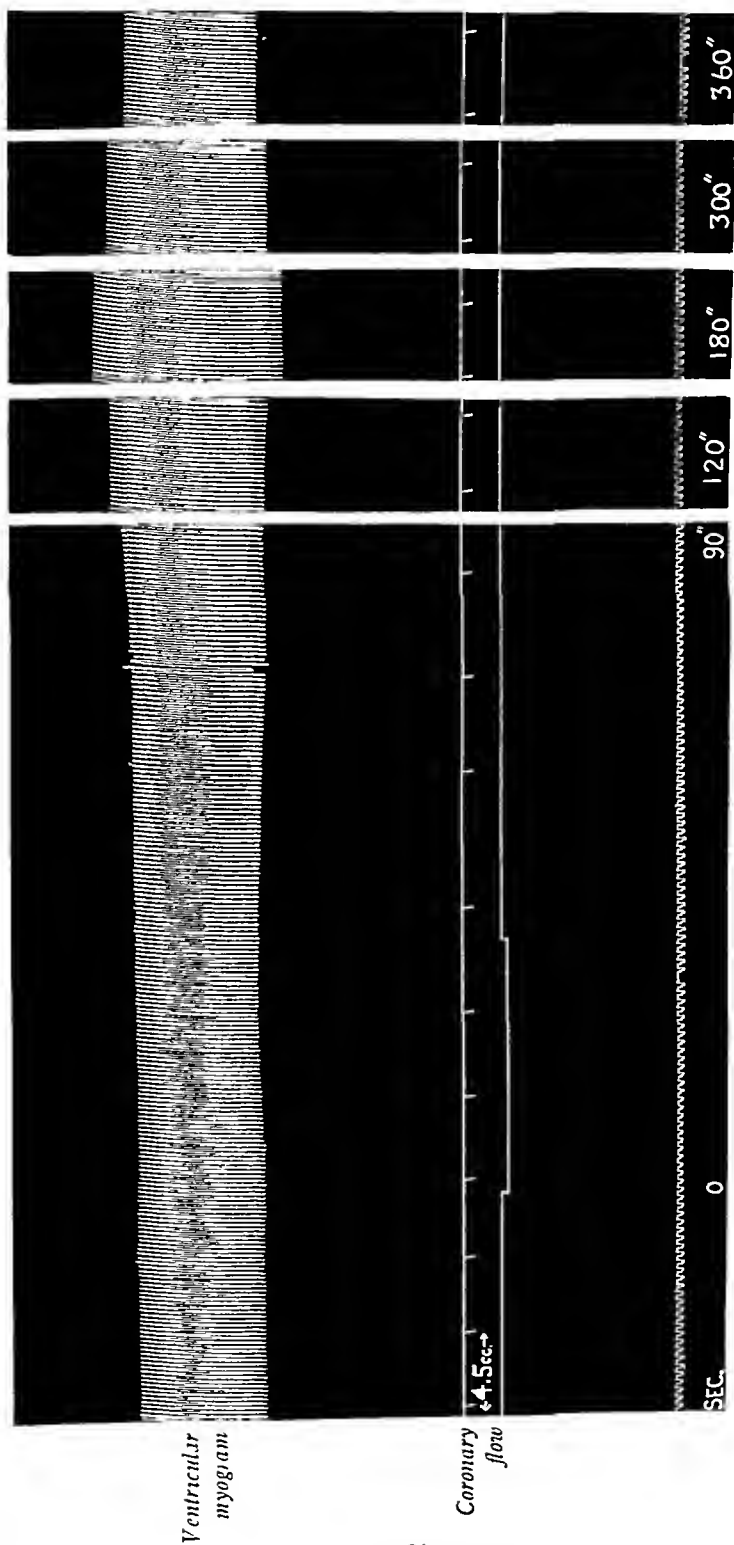


FIG 1 Isolated cat's heart perfused with Ringer-Locke solution Kymographic record of amplitude of ventricular contraction and of coronary flow Beginning at 0 over a period of 30 seconds, 0.5 cc angiotonin was injected into the stream of perfusate just above the heart

Amplitude of Beat—The amplitude of ventricular contraction, as reflected in that of the myographic tracing was augmented by every injection of angiotonin by from 18 to 300 per cent (average 92 per cent). This effect commenced later than the slowing of the coronary flow reached its maximum in 1 to 3 minutes, and persisted for 3 to 15 minutes. (See Fig 1.) The effect of angiotonin upon amplitude of beat showed no consistent tendency to diminish with successive injections. As illustrated in Fig 1, there was no visible evidence in the myographic tracing that the diastolic length of myocardial fiber shortened during the angiotonin effect.

Electrocardiogram—Records obtained by the base apex lead during the reaction to angiotonin confirmed the observation that this was associated with little or no change in cardiac rate. During and for a few minutes following, the constriction of the coronary arteries provoked by angiotonin various abnormalities appeared in the electrocardiogram, notably in the S T segment. Fig 2 illustrates the occurrence of electrical alternans during the angiotonin effect.

Effects upon the Heart Lung Preparation

Cats, weighing 2.5 to 3.5 kilos were anesthetized by the intraperitoneal injection of a solution of nembutal 35 mg per kilo. Chlorazol fast pink 80 mg per kilo was injected intravenously as an anticoagulant. One animal was bled from the carotid artery while 100 cc of warm Ringer Locke's solution was injected into a femoral vein. Using the second animal a heart lung preparation was set up after the method of Knowlton and Starling (14). Cardiac output was measured by means of a modified Gaddum (15) recorder interposed between the peripheral resistance and the venous reservoir.

In view of the results of these observations two features of the heart lung preparation deserve particular mention. Outflow from the aorta is impeded by a pneumatic 'peripheral resistance' which is uninfluenced by drugs or chemicals introduced into the circulating blood. With increase of flow through it the actual resistance offered by this device may slightly diminish but will not increase. This will act to diminish any apparent increase in arterial pressure accompanying increased cardiac output. Furthermore it should be emphasized that the level of the venous reservoir remains fixed throughout any given observation but the level of blood therein may rise or fall somewhat dependent upon the rate of flow into the heart. If cardiac output rises the rate of inflow will increase and the level of blood in the venous reservoir and

The effect of coronary constriction upon the electrocardiogram is probably somewhat exaggerated in the isolated heart perfused with Ringer Locke solution. The available oxygen supply is limited to that dissolved in the saline medium and is no doubt not far above the basal oxygen requirements of the myocardium. Any decrease of coronary flow may therefore considerably influence the spread of excitation over the heart muscle.

secondarily, venous pressure, will fall temporarily. This will mitigate against the maintenance of any increase in cardiac output.

Renin, or angiotonin, was injected into the stream of blood above the cannula inserted in the superior vena cava. The dosage of each preparation corresponded to that required to cause minimal to pronounced rise in blood pressure in a cat of 2.5 to 3.5 kilos weight. "Arterial" pressure in millimeters of mercury, and venous pressure in millimeters of saline, obtained by means of a cannula in the inferior vena cava, and the rate of flow through the Gaddum recorder, were recorded on a slow kymograph by means of calibrated Brodie bellows.

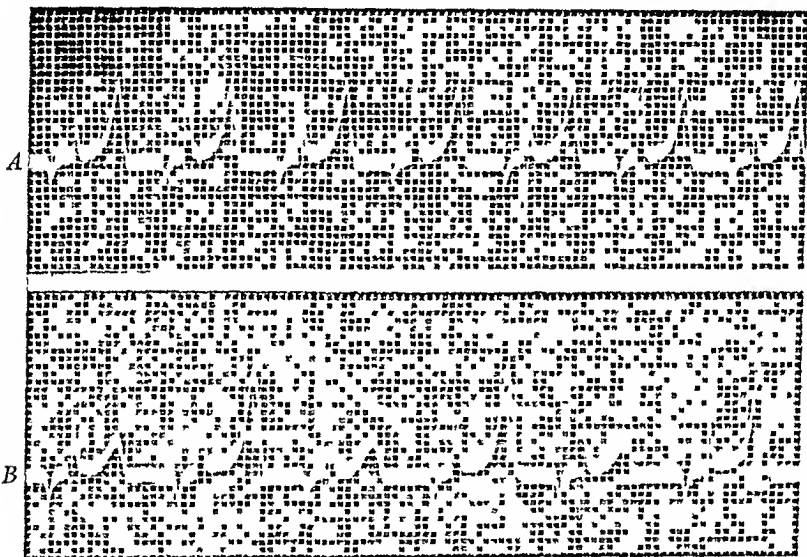


FIG 2 Electrocardiogram (base apex lead) of isolated cat's heart perfused with Ringer-Locke solution (experiment E 12). A, control normal record, rate 103 per minute. B, 3 minutes after administration of angiotonin, rate 91 per minute, electrical alternans.

Results

"Arterial" Pressure—Renin produced a measurable rise in "arterial" pressure, from 2 to 12 mm Hg, in 15 of 20 injections in 20 experiments. In two others pulmonary edema developed rapidly following the injection. The results of eight observations are shown in Table II. In these instances the effect on blood pressure lasted for from 6 to 20 minutes. Fig 3 illustrates a kymographic record of such an effect.

Angiotonin produced a somewhat more rapid, and less prolonged, rise in "arterial" pressure in 20 of 23 injections in 18 experiments (Fig 4). Twelve examples of such effects are summarized in Table II, showing rises of from 1 to 9 mm Hg lasting from 2 to 15 minutes.

Cardiac Output—The injection of renin brought about increase of cardiac output (Figs 3 and 4) of 6 to 25 per cent in 14 of 20 instances. In four observations there was no measurable effect, in two others, already mentioned, pulmonary edema rapidly developed. Eight experiments are summarized in Table II.

Angiotonin caused an increase of output (Fig. 4) in 17 of 23 injections, the maximum being 38 per cent. It brought about an increase of output followed by a decrease in three instances, and was without influence in three others.

TABLE II
Effects of Renin and Angiotonin upon the Heart Lung Preparation

Experiment No.	Substance tested	Initial pressure	Rise	Duration change	Initial output	Rise	Duration change	Initial venous pressure	Change	Duration change
	cc	mm Hg	mm	m n	cc/min	per cent	min	mm H ₂ O	per cent	m n
C 12 I	A 2 0	109	4	3	130	8	4			
C 12	A 8 0	99	4	3	79	19	4			
C 13	A 8 0	100	8	3	97	18	4			
C 14	A 8 0	85	2	5	104	7	8			
C 15	A 8 0	113	1	2	92	8	4			
C 16	A 8 0	108	4	11	73	18	10	94	-22	15
C 19	A 4 0	116	2	4	152	3	4	10	+100	10
C 20	A 2 0	113	1	5	81	38	8	128	-18	10
C 24	A 3 0	130	3	4	65	21	6	215		
C 25	A 4 0	116	9	15	104	13	15	55	-35	18
C 28	A 4 0	43	3	3	106	12	3	112	-16	3
C 29	A 4 0	98	6	10	149	8	7	60	+6	2
C 14	R 5 0	85	2	8	99	7	6	67	-14	10
C 15	R 5 0	90	12	8	65	25	20	80	-55	20
C 17	R 5 0	86	8	10	36	19	7	120	-8	10
C 19	R 5 0	102	7	15	109	12	18	115	-15	4
C 20	R 5 0	113	2	6	81	10	6	118	-8	8
C 23	R 5 0	119	2	10	125	6	10	34	+12	12
C 28	R 6 0	46	4	15	106	22	15	110	-14	10
C 29	R 6 0	102	7	20	145	9	20	74	-2	2

In reservoir

Heart Rate—Neither renin nor angiotonin exerted any apparent influence upon heart rate in the heart lung preparation. In this respect, these substances stand in sharp contrast to adrenalin, paredrine,³ or paredrinol.³ These latter increased cardiac output in this preparation even in small doses but in each instance this effect was associated with a rapid and considerable rise in heart rate.

Plethysmographic records of ventricular size were not obtained during these

³ Kindly furnished by Smith Kline and French Laboratories Philadelphia

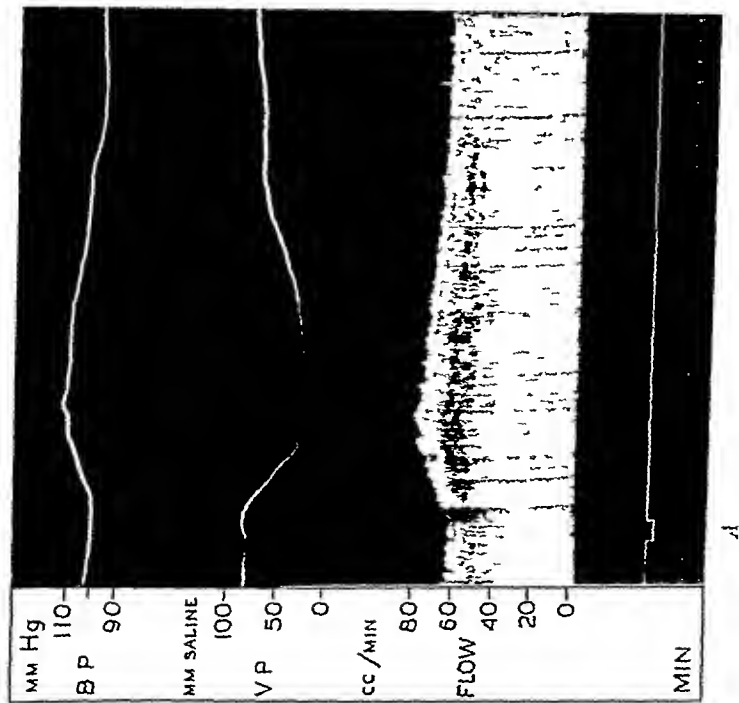


FIG 3 Effect of renin upon heart lung preparation At 1, 50 cc renin injected in 1 minute into blood stream above the heart

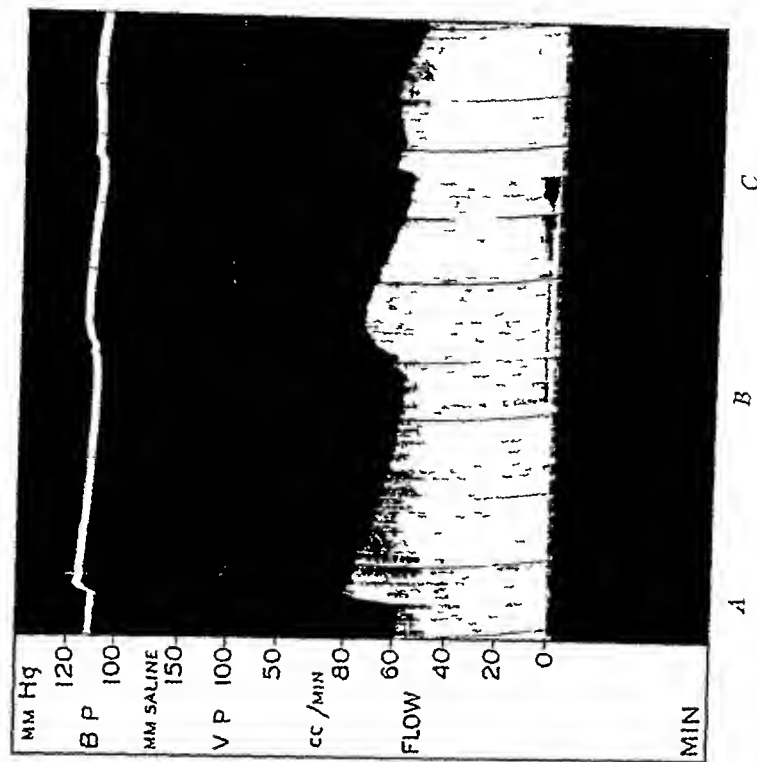


FIG 4 Effect of angiotonin and renin on heart lung preparation At A, 80 cc angiotonin injected into stream of blood above heart At B, 50 cc blood added to venous reservoir At C, injection of 5 cc renin

experiments, but it was not possible by watching the heart during this effect to observe any pronounced degree of dilatation of the ventricles. On the other hand at the height of the effect it was apparent that the amplitude of ventricular systole had considerably increased.

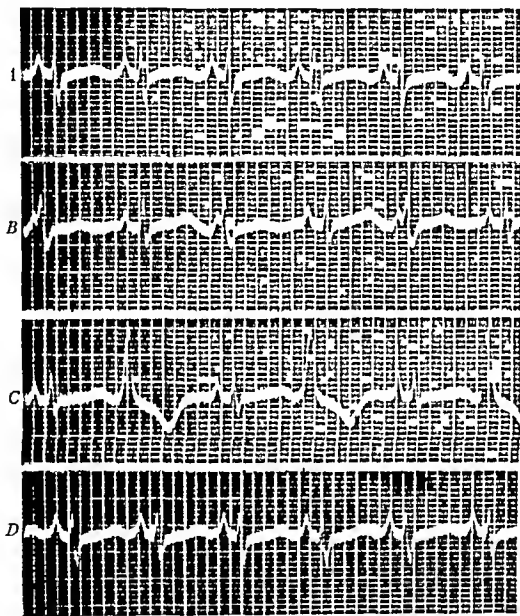


FIG 5 Cat 3 kilos Electrocardiograms (lead II) A control blood pressure 148 heart rate 200 B 3 minutes after intravenous injection of renin (2.0 cc) showing nodal extrasystoles Blood pressure 188 heart rate 127 C 2 minutes later Ventricular extrasystoles in bigeminal sequence Blood pressure 195 heart rate 200 D 20 minutes later Blood pressure 150 heart rate 209

Electrocardiograms

In eight experiments electrocardiograms (lead II—right fore leg to left hind leg) were recorded before during and at intervals after, the rise in blood pressure induced by the intravenous injection of renin or angiotonin into anesthetized cats. In no instance were changes in contour observed in these

records such as would suggest insufficient blood supply to the myocardium. The cardiac rate slowed temporarily during the pressor effect but the rhythm remained unaltered, even though the total rise in pressure was as much as 80 mm Hg, unless the level reached or exceeded 190 mm Hg. With rise in pressure above this, nodal or ventricular extrasystoles were commonly observed (Fig 5). That these were due, in part at least, to certain moderator reflexes

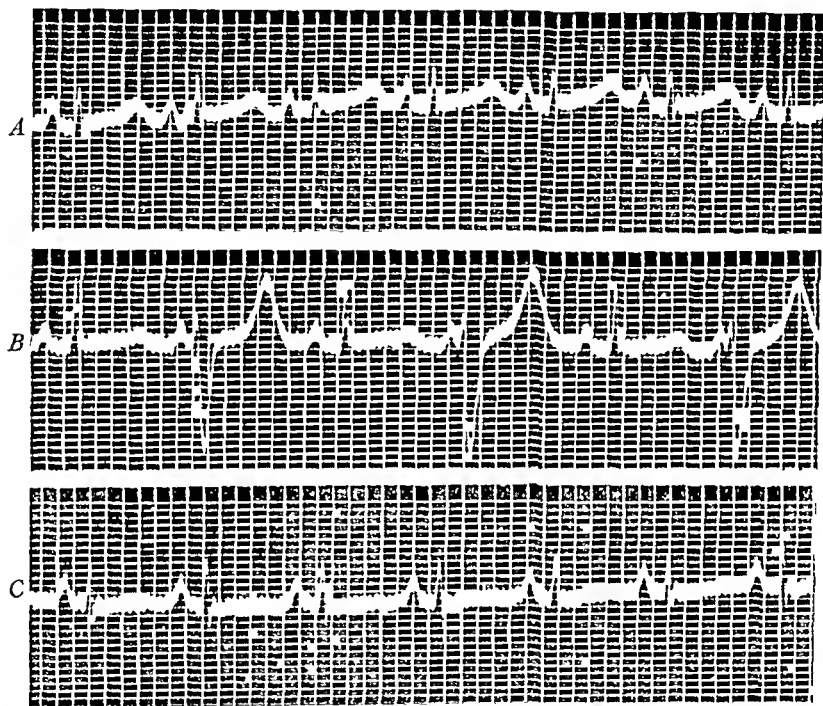


FIG 6 Cat 3.2 kilos. Electrocardiograms (lead II). A, control, blood pressure 160, heart rate 200. B, 6 minutes after injection of renin (5.0 cc). Blood pressure 210, heart rate 175. C, 2 minutes later, 1 minute after section of both vagi. Blood pressure 215, heart rate 210.

was indicated by the fact that it was possible to cause the abnormal rhythm to disappear, or to prevent its appearance, by severing both vagus nerves, or injecting atropine sulfate (Fig 6).

DISCUSSION

Under the conditions of the experiments described above it has been shown that angiotonin constricts the coronary arteries and increases the amplitude of contraction of the isolated saline-perfused heart of the cat, and, as does also

renin, augments the output and "arterial" pressure of the heart lung preparation. Certain of these conditions bear significantly upon the probable action of these substances in the intact animal. It is presumed that renin is inactive in the saline medium because of the lack of activator, the need of angiotonin activator is apparently less imperative. Moreover, it is inherent in the Langendorff method that the pressure head at the ostia of the coronary arteries is maintained constant and uninfluenced by the beat of the heart, coronary constriction is thus directly reflected in decreased coronary flow. *In vivo*, on the other hand, the results of coronary constriction may be masked, or even overbalanced, by a concomitant rise in arterial pressure. In the heart lung preparation, cardiac output and "arterial" pressure are increased by angiotonin or renin despite a constant, or even diminishing, inflow pressure and against a constant peripheral resistance. Since cardiac rate is not increased this can only mean that, under these conditions, the amplitude or vigor of ventricular systole is enhanced. In the intact animal this may be expected to oppose the influence of peripheral vascular resistance toward diminishing blood flow during the period of hypertension. There is some indication of this in the results of Landis, Montgomery, and Sparkman (16) and of Corcoran and Page (17), who observed that, in contrast with other "pressor" substances such as adrenalin and pitressin, renal extracts can cause an equivalent and considerable rise in blood pressure without so contracting the peripheral vascular field as to lower skin temperature. In these circumstances blood flow is undiminished.

The pressure existing in any fluid, such as blood, in an elastic container, such as the systemic arterial tree, will depend fundamentally upon the quantity of that fluid relative to the capacity of the container. It is unnecessary to enumerate the circumstances under which fall of systemic arterial pressure is obviously associated with underfilling of the arterial vessels (hypotension). It may bear emphasis that in all forms of hypertension either the capacity of the arterial tree is diminished by vasoconstriction, or arterial blood volume must be supposed to be increased.⁴

The results described afford evidence that the "pressor" effects of renal pressor substances are not solely due to vasoconstriction but include direct stimulation of the myocardium with increase in force or amplitude of ventricular systole. Unless, like the direct action of digitalis on the normal heart, this action of renin or angiotonin leads to diminution of ventricular volume below optimal size, the effect will be to increase cardiac output. The fact that

⁴No accurate means of measuring arterial blood volume is at present available. However, from Bazett's (18) tabulation of Mall's (19) anatomical measurements of the mesenteric vessels it is to be inferred that the arteries contain a relatively small proportion (perhaps less than 10 per cent) of the total blood in a given region of the circulation.

considerable rise in blood pressure may result without commensurate vasoconstriction may rest upon these phenomena. Potentiated, perhaps, by increase in tone of the large arteries and arterioles, even without diminution of their caliber, these effects may provoke the condition essential to arterial hypertension—the achievement of a state in which the amount of blood contained within the systemic arterial tree is increased in relation to the capacity of this arterial system.

SUMMARY AND CONCLUSIONS

Upon the isolated hearts of cats perfused with Ringer-Locke solution renin produced no significant effect. Angiotonin, on the other hand, brought about decrease in coronary flow and increase in amplitude of beat without any consistent effect upon heart rate.

Both renin and angiotonin augmented the cardiac output and raised the "arterial" pressure in the Starling heart-lung preparation, here too without influence on the heart rate.

Electrocardiograms recorded before, during and after the pressor effects of renin and angiotonin in the anesthetized cat showed no abnormalities until the blood pressure had risen above 190 mm Hg when various types of cardiac arrhythmias appeared. These were prevented, or normal rhythm was restored, by cutting the vagus nerves or injecting atropine.

It is concluded that the "pressor" effects of renal pressor substances include direct stimulation of the myocardium and augmentation of ventricular beat. Unless these actions lead to excessive decrease in diastolic volume of the ventricles, the cardiac output will be increased. The significance of this in the production of the pressor effect is discussed.

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CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

VI THE AGGLUTININ CONTENT OF ANTISERA TO HEMOLYTIC STREPTOCOCCI*

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In most of the bacterial agglutinating systems previously studied by the quantitative method (1-5) well defined capsular polysaccharide antigens and their corresponding antibodies were principally involved. The greater part of the antibody produced as a result of intravenous injections of microorganisms such as type-specific pneumococci or *H. influenzae* reacts with these capsular antigens, relatively little being directed toward minor antigens such as somatic polysaccharides and proteins.

On the other hand, immunization with hemolytic streptococci results in the production of antibodies reactive with a number of different antigenic components, of which the type specific M (6) and T (7) substances, the group specific somatic polysaccharide C (6), and at least two group-specific protein antigens (6, 8-11) have been recognized as separate entities. Adaptation of the quantitative agglutinin method to hemolytic streptococcus systems therefore raises even more complicated questions and difficulties than in the case of R (Dawson S) pneumococci (4). Before consistent results could be obtained much preliminary work was necessary and details of this phase of the study will be omitted.

Materials and Methods

The following strains¹ of *Streptococcus hemolyticus* were used:

SF 130 and S 118 both Type 1 glossy (12) strains

C203 in both the mucoid (13) and glossy phases, representative of a mixed type containing both Type 1 and Type 3 antigens (7)

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¹ For strains and assistance in phase identification the writers are indebted to Mrs. R. C. Lancefield and to Dr. M. H. Dawson and Miss Gladys L. Hobby.

Griffith's classical Type 3 strain (14) in the mucoid phase

The three glossy strains, when reexamined after passage in liquid media, were found to grow in mucoid colonies, and had thus probably reverted from the glossy type. Since it seems that reversion from smooth or glossy to the mucoid form does not necessarily involve a recovery of the M-substance,² the presence of M-substance in the reverted strains may be considered doubtful. The glossy strains were originally chosen in order to eliminate the M-anti-M system, but since it seemed difficult to preserve these strains in the glossy or smooth state, no attempt was made to isolate the large amounts needed in a state of phase purity.

Immunization—Suspensions of the strains were prepared from 24–36-hour broth cultures which were formalin-killed, centrifuged, washed free from broth with saline, and resuspended in saline. They were preserved with merthiolate,³ 1:10,000. Rabbits were injected intravenously with the vaccines, starting with doses of 0.01 mg of N, and gradually raising this to 0.15 or 0.20 mg of N. 4-week courses were given, with injections on the first 4 days of each week. 6 days after each course test bleedings were examined for presence of anti-C and antiprotein with solutions of streptococcus Group A C-substance and protein. In some instances three or four courses were necessary before satisfactory sera were obtained.

Agglutinin Determinations—The method used in the study of the first sera was the same as described previously (1–5) with minor modifications. Suspensions of the various strains were prepared from 24–36 hour cultures in 0.15 per cent glucose-meat infusion broth. The cultures were killed with formalin, centrifuged, and were washed free from broth with chilled 0.9 per cent saline. Any vaccine left standing between two experiments was centrifuged and resuspended in fresh saline to remove possible soluble extraction products. The suspensions were made up to a concentration of 0.4 to 0.6 mg of nitrogen per ml and preserved with merthiolate. In general the suspensions were found capable of removing, in the initial absorption of homologous antisera, amounts of nitrogen equivalent to 0.1 to 0.3 of the quantity of nitrogen in the streptococcus suspension used. All determinations were run in duplicate. 1.0 ml portions of 3:10 serum dilution, neutralized to pH 6.8–7.2, and 2.0 ml portions of cell suspension were usually employed. The tubes were left in the cold for 48–72 hours with occasional mixing. The agglutinated organisms and control suspensions were washed successively with 3, 2.5, and 2.5 ml of chilled saline and centrifuged in the cold.⁴

All supernatants and washings were recentrifuged in conical tubes to avoid loss of small quantities of nitrogen (4, 5). In order to exhaust the sera it was necessary to run one or more reabsorptions, and for this aliquot portions of the supernatants were used. Data obtained in this way with homologous and heterologous cell suspensions are given in Tables I and II.

Unfortunately, after a later series of streptococcus suspensions was taken into use, a serious source of error appeared in a marked tendency of the antigen to form films on the surface of the fluid and to stick to the walls of the tubes. This effect was most

² Personal communication from Miss G. L. Hobby.

³ Manufactured by Eli Lilly and Company, Indianapolis.

⁴ An International refrigerated centrifuge was used.

TABLE I

Agglutinin N Content of Antistreptococcus Rabbit Sera at pH 6.8-7.2 and Diluted 4:10 by Absorption with SF 130 (Type 1, Glossy) Suspension

Absorption	Serum	Volume of dilution used	Bacterial N used	Total N precipitated	Agglutinin N		
					Found	Calculated to 1.0 ml of undiluted serum	Total per 1.0 ml of undiluted serum
		ml	mg	mg	mg	mg	mg
1st	705-12*	1.0	0.776	0.996	0.220	0.55	
2nd		Supernatant $\times 5/6$	0.780	0.872	0.092	0.28	
3rd		$\times 7/8$	0.796	0.836	0.040	0.14	0.97
1st	700 ₂ †	1.0	0.784	0.884	0.100	0.25	
2nd		Supernatant $\times 4/5$	0.784	0.820	0.036	0.11	
3rd		$\times 6/7$	0.780	0.806	0.026	0.09	
4th			0.920	0.912	0	0	0.45

From rabbits injected with strain SF 130 (Type 1 originally glossy)

† From rabbit injected with strain C203 mucoid. Subnumeral indicates course of injections

TABLE II

Agglutinin N Determinations in Diluted Antisera to Type 1 Glossy (SF 130 gl) and C203 Hemolytic Streptococcus with Homologous and Heterologous Suspensions

Serum	Strain used for production	Agglutinin N removed by suspension used for analysis			
		SF 130 glossy	C203 mucoid	C203 glossy	Type 3 mucoid
		mg	mg	mg	mg
710 ₁	SF 130 gl	0.40	0.25		
710 ₂		0.60	0.50	0.53	
710 ₃		0.88		0.48	0.23
705-12		0.97		0.83	{ 0.52 0.38*
705-12†		0.59		0.41	0.34†
	C203 M	{ 1.67§ 1.44 1.48		{ 1.05 1.25	0.88
700 ₂		0.45		0.51	
103-07		{ 0.48 0.60	{ 0.41 0.43 0.54		
707 ₂		0.74	0.71		0.27

Subnumerals indicate course of injections

* Repeated absorptions of 1.0 ml of undiluted serum

† Repetition 1 year later

‡ Removal of agglutinins not quite complete after 2nd absorption

§ Removal of agglutinins not quite complete after 4th absorption

|| Removal of agglutinins not quite complete after 3rd absorption

TABLE III

Effect of Washing Blank Suspensions, and Addition of Saponin to Washings

Absorption	Suspension used	Suspension N added			Total N pre- cipitated D 1.0 ml serum 705 12(3 10) + suspension	Agglutinin N		
		A Unwashed suspension	B Suspension washed 3 times	C Suspension washed with saponin saline		D A	D B	D C
		mg	mg	mg	mg	mg	mg	mg
1st	SF 130	0 94	0 90	0 92	1 03	0 09	0 13	0 11
2nd	"	0 88	0 86	0 88	0 95	0 07	0 09	0 07
1st plus 2nd						0 16	0 22	0 18
1st	S 118	0 88	0 85	0 86	0 95	0 07	0 10	0 09
2nd	"	0 79	0 76	0 77	0 81	0 02	0 05	0 04
1st plus 2nd						0 09	0 15	0 13

Differences between A and C were checked by analysis of the cell free supernatant from C

TABLE IV

*Agglutinin N Estimations on Rabbit Antisera to Streptococcus hemolyticus, with Use of Saponin
1.0 Ml of 3.10 Serum Dilution Used*

Serum	Strain used for immunization	Agglutinin N per ml of undiluted serum taken out by			Type specific agglutinin N†
		SF 130 gl Type I	Type 3 M	SF 130, R*	
		mg	mg	mg	mg
750 ₁	SF 130, gl	0 29	0 16		0 13
750 ₂	"	0 42	0 26	0 42	0 16
750 ₃ †	"	0 35	0 22		0 13
752 ₁	"	0 23	0 09		0 14
752 ₂	"	0 41	0 25	0 50	0 16
752 ₃ †	"	0 47	0 37		0 10
753 ₁	"	0 29	0 15		0 14
756 ₁	"	0 23	0 12		0 11
756	"	0 40	0 32	0 43	0 08
756 ₃ †	"	0 51	0 36		0 15
758 ₁	Type 3	0 08	0 35		0 27
758 ₂	"	0 17	0 49	0 26	0 23
759 ₁	"	0 28	0 36		0 08
759 ₂	"	0 09	0 29	0 07	0 20
759 ₃ †	"	0 27	0 51		0 24

Subnumerals indicate course of injection. Sera from the first two courses were given two absorptions only, agglutinin N removal was probably incomplete in 750, 752₂, 756₁, 756₂, and 758. Sera from the third course were given three absorptions, of which the first two sufficed for serum 750₃.

* Suspension prepared from R strain, broth culture showed typical properties of R, but smears indicated possible presence of 5 to 10 per cent of S organisms.

† Homologous agglutinin N minus heterologous agglutinin N. Both include the anti C

† Analyses by Mr. Manfred Mayer. A pool of approximately equal quantities of 750₁, 752₁, and 756₁ contained 0.15 mg of anti C N per ml.

pronounced in the suspension control tubes and rarely appeared in the tubes which had contained serum until the second washing resulting in greater loss of nitrogen in the suspension N estimations than in the analyses of the agglutinated suspensions. This tended to make the results too high. If the suspension N estimations were made without washings parallel with those on the agglutinated cells the values for agglutinated suspension N minus control suspension N were occasionally 50 per cent less, for low grade sera, than if washed suspensions were used for the N value of the cell suspension. However, it was found that a better method of washing was es

TABLE V

Precipitins for Group A Specific Carbohydrate (C Substance) in 10 Ml Undiluted Antistreptococcus Sera Compared with Total Agglutinins

Serum	Type	Amount of C-substance used	Precipitin N	Agglutinin N
		mg	mg	mg
703-07	C203	0.035	0.17	0.61
707 ₂		0.035	0.17	0.80
700 ₁		0.09	0.19	
700 ₂		0.04	0.16	0.50
703 ₁		0.03	0.11	0.67
706 ₁		0.07	0.18	
708	1	0.025	0.09	0.81
705-12		0.05	0.18	0.96
713		0.06	0.32	1.67
710 ₁		0.025	0.13	0.40
710 ₂		0.025	0.11	0.61
710 ₃		0.025	0.10	0.88
1870*	Polyvalent	0.1	0.35	

* Horse serum E. R. Squibb and Sons bleeding of Mar. 6, 1940. After removal of anti C the supernatant gave no precipitate with a number of protein fractions.

sential, since other experiments had shown that the cell free washings of the control suspensions always contained small amounts of nitrogen. It therefore seemed possible that similar small amounts would be lost from the agglutinated cells during the washings, particularly in the second and third absorptions after the major portion of the agglutinin N had been removed from an antiserum.

In view of the effect of serum in preventing film formation by the streptococci it was thought that a non nitrogenous substance such as saponin might likewise be effective. Addition of 1 or 2 mg of this substance to the control suspensions resulted in a greater loss of soluble N than with saline alone, even though film formation was prevented while addition of even 0.5 mg per ml to the entire suspension resulted in an unusually low value for agglutinin N. The following procedure eliminated film formation as well as these disturbing effects of the saponin and may be recommended as a standard technique. Saponin is added only to the supernatants which contain only small numbers of cells.

heterologous types or preliminary precipitation with C-substance (16) proved to be unnecessary

In repeated absorptions of supernatants the heterologous agglutination was the first to disappear, while homologous agglutination was evident as long as measurable quantities of antibody nitrogen were bound. Occasionally, type-specific agglutination persisted even after the amount of agglutinin nitrogen bound was within the analytical error of 0.02 to 0.04 mg of nitrogen, indicating that a minute amount of antibody may suffice to agglutinate relatively large quantities of homologous suspension. Two portions of anti-SF 130 serum 713 with an agglutinin N content of 1.66 mg per ml for the homologous strain, and an agglutinin "titer" of about 1:15,000 were absorbed with SF 130 and C203, respectively, until practically all determinable antibody nitrogen was removed. The supernatants agglutinated the same suspensions, by the ordinary qualitative method, to titers of 100 to 500, while a Type 3 suspension was not agglutinated.

Antibodies to C-substance are among those most easily removed from the sera (see also Table V) since supernatants failed to react with C, while absorbed sera that no longer gave measurable quantities of agglutinin N still showed weak precipitation with the most active streptococcus protein fractions. Table V also shows that the proportion of anti-C in the rabbit antisera tested was low, in contrast to the pneumococcus anti-C in certain antisera to R (Dawson S) pneumococci (4).

Two specific precipitates, calculated to contain an excess of antibody, were prepared, one from an unabsorbed antistreptococcus serum and streptococcus C-substance,—the other from C-absorbed serum and a streptococcus protein, fraction 51D' (17) (similar to D-fractions in references 8–10). The precipitates were washed five times with chilled saline, resuspended as evenly as possible in saline, and tested with suspensions of SF 130 glossy, C203 glossy, and Type 3 hemolytic streptococci. The conditions of this experiment were analogous to those of the reagglutination experiments reported in reference 3 in which Type 1 pneumococci, agglutinated in fine floccules by excess antibody and resuspended in saline, were reagglutinated into large floccules when unsensitized Type 1 pneumococci were added, owing to chemical combination of free antibody groupings on the sensitized cells with specific polysaccharide on the unsensitized cells. Immediate agglutination of all three streptococcus cell suspensions also took place when these were mixed with the C-anti-C and protein-anti-protein precipitates, showing directly that Group A anti-C and antibody to the protein fraction take part in the agglutination of these strains of hemolytic streptococci. With respect to the anti-C this agrees with the tests showing disappearance of this antibody after removal of agglutinins. Evidently not all of the antiprotein is equally reactive.

These experiments may help to clarify somewhat the complicated status of

type specific agglutination of Group A hemolytic streptococci. They are in accord with Lancefield's demonstration that type-specific agglutination is brought about by antibodies to the M- and T antigens, although they furnish no new or direct evidence in this regard. On the other hand, they show clearly that antibodies to the C carbohydrate are taken up in type specific as well as group-specific agglutination of the streptococci, and that these antibodies, when suitably isolated, are capable of agglutinating cells of both homologous and heterologous types. The conclusions of Pauli and Cohurn (16) regarding cross agglutination due to anti C are therefore substantiated by these two sets of tests along entirely different lines. It would appear, however, from the data in Tables IV and V that sera containing only a small proportion of type specific antibody would show less improvement in type specificity on removal of anti C than sera in which the antibody is largely type specific.

On the whole, the quantitative data on streptococcus agglutinins are somewhat less accurate and more difficult to interpret than those in the other systems studied (1-5). Reproducible results are most easily obtained when the analyses are carried out within a short period and with the same cell suspensions. Both the immune sera and the bacterial suspensions seemed to change unpredictably. The proportions of the reagents also seemed of some importance since multiples of the small quantities of serum usually used rarely yielded the expected quantity of antibody nitrogen. Nevertheless, the method with all its limitations, still appears capable of giving more precise information (to the nearest tenth of a milligram of antibody nitrogen per milliliter of antiserum) regarding the serological properties of the various types and phases of hemolytic streptococci and their antisera than is obtainable by the relative and essentially qualitative methods hitherto in use.

SUMMARY

1 Application of the quantitative agglutination procedure to hemolytic streptococci and their antisera is shown to yield values indicative of the antibody content of the antisera in weight units.

2 Estimations are given of type-specific and group-specific antibody in a number of antisera.

3 An incomplete analysis is given of the antigenic components and antibodies involved in the agglutination.

4 Adaptation of the experimental conditions to a simple qualitative type determination of hemolytic streptococci is suggested.

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intramuscularly injected virus fails to produce encephalitis in most of the animals, but in the exceptional ones which succumb the virus is found to have entered by routes other than the olfactory. In each case, however, the virus travels along a closed nerve pathway. In a comprehensive study of vesicular stomatitis virus, Sabin and Olitsky (8*b*) found that virus injected intramuscularly in the leg muscles of young mice multiplies locally and invades the sciatic nerve and spinal cord. In old mice, no such local multiplication occurs and, although the active virus persists, no invasion of the sciatic nerve nor of the central nervous system takes place.

Age is known to be a factor in determining also the degree of immune response of which an animal is capable.

As early as 1897, Metchnikoff (10) found that when alligators are injected with tetanus toxoid large animals respond with a higher concentration of antitoxin in their serum than small ones. Freund (11) demonstrated an increasing antibody production with increasing age of rabbits in response to a variety of antigens: formalized typhoid bacilli, sheep red cells, horse serum, and egg albumin. Baumgartner (12) presented a thorough review of the subject up to 1932. In studies on *Trypanosoma lewisi*, Culbertson and Kessler (13*a*) observed that with increasing age of rats a greater antibody response as well as greater resistance to living trypanosomes develops. Kolodny (13*b*) added data on a greater rate of antibody formation with age. Morgan (14) found that the ability of mice to be immunized with formalized virus of Eastern equine encephalomyelitis increases with age, as tested by cerebral resistance, as well as by degree and rate of antibody formation. Casals (15) reported diminishing susceptibility with age of mice to peripheral injection of rabies virus and an increasing immunizability with age.

The experiments to be reported here were carried out to find what relationship exists between the influence of age of mice on their susceptibility to peripheral introduction of the virus of Eastern equine encephalomyelitis and on their capacity to elicit an immune response.

Methods and Materials

Active Virus—Mouse brain infected with the virus of Eastern equine encephalomyelitis (E E E) was used in the experiments. This strain was obtained 6 years ago from Dr. C. TenBroeck and has been through more than 200 mouse brain passages in this laboratory. The Western strain of equine encephalomyelitis virus (W E E) was obtained 8 years ago from Miss B. Howitt. Stock virus, dried from the frozen state by the Flosdorf-Mudd lyophilic method, was kept *in vacuo* in rubber-stoppered glass vials in the refrigerator. Before use, it was passaged intracerebrally in normal mice, and the brains of at least three mice prostrate or recently dead were removed at autopsy. These were weighed and ground without abrasive, meat infusion broth at pH 7.4 was added to make a 10 per cent suspension. After light centrifugation, 5 minutes at 2,000 R P M, the supernate was removed. This was designated 10^{-1} E E E virus, tenfold dilutions made in broth from this were then 10^{-2} , 10^{-3} , etc.

Formalin Inactivated Virus—To a 20 per cent broth suspension of mouse brain infected with E C E virus was added an equal volume of 1 per cent formalin (containing 37 per cent formaldehyde) in 0.85 per cent NaCl solution thus making a 10 per cent infected mouse brain suspension in 0.5 per cent formalin. The preparation was kept at room temperature in the dark for 2 days and then placed in the refrigerator at 4°C. For a vaccine to be acceptable the virus, before the addition of formalin must have been infective to a titer of 10^8 when injected intracerebrally into normal mice. After a week in the refrigerator, and again when first used for vaccination the vaccine was tested intracerebrally in mice to determine whether all active virus had been destroyed. It may be stated that by the most stringent tests we have never been able to detect active virus in such a formalized preparation.

Mice—Albino mice, Rockefeller Institute strain, were used throughout. In the stock colony, the date of birth of each litter of mice is recorded. Young of the stock mice are weaned at 21 days of age, in the course of experiments they were frequently weaned at 14 days. In the stock colony, mothers with young are fed purina fox chow pellets and given water from a water bottle. When young are weaned, a slice of bread soaked in water is added daily to the pellet diet. When mice are taken from the stock, and kept for an experiment, an equal part of pasteurized grade B milk is added to the water in which the bread is soaked. Pellets are still in the diet, but the amount needed is less. The average weight of young mice of various ages used in the following experiments is given below.

Age days	2	5	7	10	13	15
Average weight gm	2.0	3.5	4.9	5.5	8.5	10.6
No of mice as basis for average weight	76	107	118	22	24	28

Routes of Injection—Intracerebral. A short incision was made to one side of the midline in the scalp of a mouse under ether anesthesia. With a fine trephine a hole was bored through the skull in the angle formed by the sagittal and fronto-parietal sutures about 2 mm from each, through this 0.03 cc dilution of virus was injected. In younger mice the trephine was not used. Accidental deaths as a result of this method of injection were very few.

Intraperitoneal. The needle was inserted subcutaneously for a short distance and then plunged into the peritoneal cavity, on removal, the needle was gently turned to draw the skin together at the site of injection. Various doses were used in the intraperitoneal injections but it may be mentioned that even the youngest mice used, 2 days old could retain 0.1 cc. of injected vaccine with little loss.

Intramuscular. 0.1 cc injections of vaccine were made into the muscles of the thigh of one hind limb. This dose was well retained.

Bleeding—When relatively large amounts of blood were needed anesthetized mice were bled from the heart. If it was necessary for mice to survive they were bled by snipping off a piece of tail. Sufficient blood was obtained, even from young mice, provided they had been kept in a warm place before bleeding.

Intraperitoneal Injection of Active Eastern Equine Encephalomyelitis Virus in Mice of Various Age Groups

The susceptibility of mice of various ages to the intraperitoneal injection of active E E E virus was tested. Since the last report from this laboratory (8) on susceptibility of mice to E E E virus given by the peritoneal route, two changes have taken place: (1) The virus has been through a greater number of mouse brain passages, and (2) the average weight of mice of a given age has gradually increased.

Groups of mice 14 days, 22 days, 1 month, and 6 months of age were injected intraperitoneally with 0.25 cc 10^{-3} dilution of active E E E virus. The amount of virus, when tested intracerebrally in normal adult mice, proved to be 1,000,000 cerebral lethal units. The outcome is shown in Fig. 1.

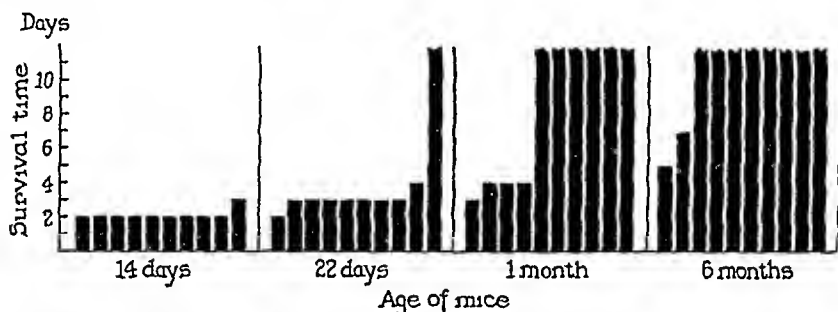


FIG. 1. Susceptibility of mice of various ages to intraperitoneal injection of active E E E virus (1,000,000 cerebral units).

None of the ten mice 14 days old survived the intraperitoneal injection of active virus. One of ten, 22-days-old, six of ten, 1-month-old, and eight of ten, 6-months-old mice, survived to the end of the 12-days observation period. Deaths due to virus have not been noted beyond this time. With increasing age, the average incubation period of those which succumbed was 2, 3, 4, and 6 days respectively. Incubation period is used in the sense of time between injection and death, which followed closely upon first symptoms of central nervous system involvement. It should be added that the incubation period for any given age is shorter after intracerebral than after intraperitoneal injection of active virus.

With increasing age, mice showed a diminishing susceptibility to intraperitoneal injection of active virus. Of those which succumbed, the length of incubation period increased with age. These results are consistent with previous reports (7 b, 8 a).

Antibody Response to Active Virus in Mice of Various Age Groups

The antibody response of mice of various ages to intraperitoneal injection of active E E E virus was studied, in order to determine whether the age of

the surviving mice affected the amount or rate of formation of serum neutralizing antibodies

Additional groups of mice of the ages shown in Fig 1 were injected intraperitoneally with the same dose of 0.25 cc 10^{-3} dilution of E.E.C. virus. Surviving mice were bled from the heart on the 3rd, 4th, 5th, and 8th days after injection. Sera were pooled according to age and time after injection; each pool contained sera from at least three mice. There was not a sufficient number of survivors of the 14-days-old group to bleed on the 3rd day, nor of 22-days-old mice after the 3rd day. Test for antibody was carried out by the intraperitoneal serum neutralization test of Olitsky.

Final dilution of virus I.P.	Normal serum	Sera No. of days after injection							
		3		4		5		6	
		Serum of mice aged							
		22 days	1 mo	6 mos	1 mo	6 mos	1 mo	6 mos	
10^{-2}					■	■	■	■	
10^{-3}					■	■	■	■	
10^{-4}				■	■	■	■	■	
10^{-5}	■	■	■	■	■	■	■	■	
10^{-6}	■	■	■	■	■	■			
10^{-7}	■	■	■	■	■				
10^{-8}	■	■	■	■					

■ 1 mouse died. □ 1 mouse survived

■ 1 mouse died □ 1 mouse survived

FIG 2 Development of serum neutralizing antibodies in mice of various ages in response to intraperitoneal injection of active E.E.C. virus

and Harford (16). 0.03 cc. of each mixture of serum and dilution of virus was injected intraperitoneally into each of four mice. The ratio of serum to virus dilution was 4 to 1; for example, 0.2 cc of serum was mixed with 0.05 cc of virus dilution (final dilutions of virus are recorded), 0.03 cc of this mixture was injected. The results are given in Fig 2.

The titer of virus in the presence of normal serum was 10^7 , and was 10^6 in the presence of serum taken on the 3rd day from 22-days-old, 1 month-old, and 6-months-old mice. This difference in titer does not, however, establish the presence of antibody at this time. In our experience a difference in titer must be more than tenfold to be significant when so few mice are injected. The essential point is that there was no difference in protective capacity of the three test sera. On the 4th day, the sera of the 1 month-old and 6-months-old mice showed perhaps a slight protective effect, but again no difference was apparent between the test sera. By the 5th day, the sera of the 1 month-old and 6-months-old mice protected about half of the mice at dilu

tions of virus ranging from 10^{-2} to 10^{-5} . It may be noted that this protection of half of each group over a range of virus dilutions, rather than the clear-cut end point found with very strong or very weak antisera, is characteristic of a serum of moderate antibody content. By the 8th day, sera of mice of the two surviving age groups protected at least half the number of mice which received the 10^{-2} dilution of virus, or 100,000 peritoneal units of virus, as compared with the normal serum control.

Thus, there was no difference demonstrable in antibody response of *surviving* mice of various age groups after intraperitoneal injection of active virus. However, it must be kept in mind that there is inherently a selection of mice available for this antibody study. The survivors are the more resistant animals of each group and, in the younger groups especially, are hardly representative. Expressed in another way, mice surviving an intraperitoneal injection of active virus, regardless of age, were indistinguishable in rate of antibody development.

Immune Response of Young Mice to Formalized Virus

Since there were difficulties inherent in the direct approach to the study of immune response to active virus with increasing age, a more indirect approach was resorted to, by vaccinating young mice with inactivated, formalin-treated virus. In a previous study of immune response with age (14), it had been observed that although 2-days-old mice, after vaccination with formalin-treated virus, exhibited little or no antibody and no cerebral immunity, nevertheless they resisted 1,000 times more virus introduced intraperitoneally than their non-vaccinated litter mates. This finding was investigated further in the following experiments.

Immunization had to be achieved before the mice were 15 days old, since beyond that age normal mice rapidly lose their susceptibility to peripheral injection of the virus. Using an interval of 8 days between the beginning of vaccination and test with active virus, it was possible to start vaccination when mice were 2, 5, and 7 days old and test for resistance with active virus when 10, 13, and 15 days old. Mice of these age groups were vaccinated with a 10 per cent EEE virus-infected mouse brain suspension in 0.5 per cent formalin in which no active virus was detectable by cerebral test. They were injected intraperitoneally with three doses of 0.1 cc. of inactivated virus on alternate days. This dosage was well retained, even by the youngest animals. Since all mice received the same amount of vaccine, the ratio of dose to body weight was smaller in the larger animals. However, since it has already been shown (14) that the immune response of older mice is greater in spite of the proportionally small amount of antigen, the unequal dosage per body weight may be disregarded.

Cerebral and Peritoneal Resistance—A comparison was made between the degree of peritoneal and of cerebral resistance induced by intraperitoneal vaccination with formalin-inactivated virus.

Eight days after the beginning of vaccination, at least six vaccinated mice of each age group were bled from the heart and the sera pooled according to age. Of the remaining vaccinated mice some were tested for resistance to active virus injected intracerebrally and some intraperitoneally. Non vaccinated litter mates served as controls for each route of injection. The results presented in Fig. 3 are based on data from three experiments, each of which included all groups.

Route of active virus	Dilution of virus	Age of mice at beginning of vaccination		
		2 days	5 days	7 days
IP	Vaccinated mice			
	10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}			
	Infective titer	3.2×10^5	4.5×10^3	1.4×10^2
	Non vaccinated litter mates			
IC	10^{-5} 10^{-7} 10^{-8} 10^{-9}			
	Infective titer	2.2×10^8	1.7×10^7	1.9×10^6
	Peritoneal resistance	690	3800	13,500
	Vaccinated mice			
IC	10^{-5} 10^{-6} 10^{-7} 10^{-8}			
	Non vaccinated litter mates			
	10^{-7} 10^{-8} 10^{-9}			

■ = 1 mouse died □ = 1 mouse survived

FIG. 3. Resistance to intraperitoneal and intracerebral injection of active EEE virus after intraperitoneal vaccination with formalized EEE virus.

The cerebral titer of EEE virus as measured by the 50 per cent end point of death in normal 10, 13, and 15 days-old mice, was 10^8 . This titer was not changed as a result of vaccination of mice of the same ages. In mice 10, 13, and 15 days old the 50 per cent end point of virus injected intraperitoneally, as calculated by the Muench accumulation method was 2.2×10^8 , 1.7×10^7 , and 1.9×10^6 , respectively. As a result of vaccination begun 8 days previously, when mice were 2, 5, and 7 days old the peritoneal titer was 3.2×10^5 , 4.5×10^3 , and 1.4×10^2 , respectively indicating a peritoneal resistance, as measured by difference in titer, of 690, 3,800 and 13,500 each compared with its non vaccinated control. The lack of sharp end point in the vaccinated, intermediate age group, which was apparent in each of the tests

contributing to this figure, recalls the partial protection, over a wide range of virus dilutions, of groups of mice receiving virus plus serum containing only a moderate amount of antibody (Fig 2, 5th-day serum). This partial group resistance may be an expression of the differences between individuals which become apparent at this intermediate level of resistance of the group. It may be added that mice which succumbed in spite of vaccination died after the shorter incubation period characteristic of young non-vaccinated mice rather than that of adult mice after intraperitoneal injection of active virus.

The titer of E E E virus injected intracerebrally was the same in mice of the ages studied. Vaccination with formolized virus failed to induce cerebral resistance. Although the susceptibility of normal mice to intraperitoneally injected E E E virus decreased slightly with age from 10 to 13 to 15 days, as measured by titer of virus, the amount of peritoneal resistance induced by formalin-treated E E E virus increased progressively with these small increments of age. The susceptibility of the young vaccinated mice recalls that of normal adult mice, that is, although fully susceptible to virus injected cerebrally, the majority resisted peritoneally many times the amount of virus fatal to non-vaccinated young mice.

Antibody Response—Sera of mice vaccinated at the same time as those tested for cerebral and peritoneal resistance were studied for neutralizing antibody.

Sera taken 8 days after the beginning of vaccination of mice 2, 5, and 7 days of age were tested for capacity to neutralize E E E virus. The intraperitoneal serum-neutralization test (16) was used. Mice 13 to 14 days of age were injected intraperitoneally with 0.03 cc. of mixtures of equal parts of serum and virus dilutions. The results reported in Fig 4 represent five different tests, each of which included all three test sera as well as control normal serum. 50 per cent end-points were calculated by the Muench accumulation method.

The difference in titer of virus in the presence of test serum and that in normal serum was taken as the measure of neutralizing capacity of the serum. These were found to be 5.9, 25.6, and 123 respectively for sera of mice 2, 5, and 7 days old at the beginning of vaccination.

Correlation of Peritoneal Resistance with Antibody—When the logarithm of the peritoneal resistance induced by vaccination of mice 2, 5, and 7 days old was plotted, as in Fig 5, and compared with the logarithm of neutralizing capacity of a sample of serum, they were found to be almost parallel. The ratio of peritoneal resistance to neutralizing capacity of a serum sample varied from 110/1 to 148/1. Thus antibody content, as measured by serum-neutralization tests, paralleled peritoneal resistance at a lower level and was therefore found to be a less sensitive measure of immune response than intraperitoneal inoculation of active virus. The fact that the degree of peritoneal resistance

One group of 7-days-old mice was vaccinated by intraperitoneal injection of formalized virus as before, and another group by intramuscular vaccination. The mice were injected three times on alternate days with 0.1 cc. of formalin-inactivated EEE mouse brain virus suspension. Active virus was injected into half of each vaccinated group by the same route as that used for vaccination and half by the other route. The results of two experiments are presented in Fig. 6.

Mice vaccinated intraperitoneally were no better protected against active virus injected by the intraperitoneal than by the intramuscular route, and

Dilution of active virus	Vaccinated mice			
	Intramuscular (IM)		Intraperitoneal (IP)	
	Route of active virus			
	IP	IM	IP	IM
10 ⁻¹			■■■■	■■■
10 ⁻²	■■■■■■■■■	■■■■■■■■■	■■■■■■■■■	■■■■■■■■■
10 ⁻³	■■■■■■■■■	■■■■■■■■■	■■■■■■■■■	■■■■■■■■■
10 ⁻⁴	■■■■■■■■■	■■■■■■■■■	■■■■	■■■■
	Non vaccinated litter mates			
	Route of active virus			
	IP		IM	
10 ⁻⁵	■■■■■■■■■■■		■■■■■■■■■■■	
10 ⁻⁶	■■■■■■■■■■■		■■■■■■■■■■■	
10 ⁻⁷	■■■■■■■■■■■		■■■■■■■■■■■	
10 ⁻⁸	■■■■■■■■■		■■■■	

■=1 mouse died □=1 mouse survived

FIG 6

Dilution of active virus IP	4th day	5th day
Vaccinated mice		
10 ⁻³		■■■
10 ⁻⁴	■■■	■■■
10 ⁻⁵	■■■	■■■
10 ⁻⁶	■■■	
Normal litter mates		
10 ⁻⁵	■	■■■
10 ⁻⁶	■■■	■■■
10 ⁻⁷	■■■	■■■

■=1 mouse died
□=1 " survived

FIG 7

FIG 6 Comparison of resistance to active EEE virus by intraperitoneal and by intramuscular routes after intraperitoneal or intramuscular vaccination with formalized EEE virus.

FIG 7 Immunity to active EEE virus by the intraperitoneal route 4 to 5 days after beginning of vaccination of 10-days-old mice with formalized virus.

conversely, in mice vaccinated intramuscularly the resistance to active virus given by the same route was no greater than to virus by the intraperitoneal route. Thus, the resistance induced by vaccination proved to be not local, but a general, systemic immunity.

Time of Appearance of Immunity to Active EEE Virus Given by the Intraperitoneal Route—It was desirable to choose mice as old as possible for vaccination, since the immune response has been shown to increase with age, yet of such age that control litter mates at the time of test would still be susceptible to active virus by the peritoneal route.

With these limitations in mind, vaccination was begun when mice were 10 days of age. Half of the number of mice born on a given day were vaccinated, the others remained as non-vaccinated controls. Vaccination consisted of three intraperitoneal injections of 0.1 cc. of formalin-inactivated EEE virus on consecutive days. Tests

for resistance to active E.E.E. virus injected intraperitoneally were carried out on the 2nd, 3rd, 4th, and 5th days after the beginning of vaccination. 4th and 5th-day groups were tested simultaneously, vaccination having been begun on consecutive days when the mice in each group were 10 days old. The young mice were kept with their mothers until tested with active virus when they were, respectively, 12, 13, 14, and 15 days of age.

Immunity to active virus by the peritoneal route was demonstrable on the 4th and 5th days after beginning of vaccination, as charted in Fig. 7.

The difference between the titer of virus in non-vaccinated mice and that in vaccinated mice on the 4th day was at least 1,000-fold, on the 5th day, more than 10,000-fold. Other experiments indicated a small amount of resistance on the 2nd and 3rd days. However, this proved to be non-specific when mice vaccinated with the Eastern strain were compared with Western-vaccinated as well as with non-vaccinated control mice. The specificity of the immunity present on the 4th day was tested in the following experiments.

Specificity of Immunity Induced by Vaccination with Formalized W.E.E. or E.E.E. Virus—The Western strain of equine encephalomyelitis virus, the virus most closely related to the Eastern strain, is known to be immunologically distinct from the Eastern, when compared in artificially immunized laboratory animals (17 a) as well as in convalescent horses (17 b). Therefore the specificity of the immunity induced by vaccination with formalized Eastern strain compared with that by the Western was tested by intraperitoneal injection of active virus of the homologous or heterologous strain.

One third of a large group of 10-days-old mice was vaccinated with formalized Eastern strain, one third with formalized Western, and a third left as non-vaccinated controls. Vaccination was carried out by three intraperitoneal injections of 0.1 cc of formalin-inactivated virus on consecutive days. On the 4th day after the beginning of vaccination, when mice were 14 days old, they were tested by the intraperitoneal injection of active Eastern strain of virus.

In another experiment, one third of a group of 7-days-old mice was vaccinated with formalized Eastern strain, one third with formalized Western, and the remainder kept as controls. Vaccination consisted of three intraperitoneal injections of 0.1 cc of formalized virus on alternate days. 8 days after the first injection of vaccine the mice, then 15 days old, were tested with active W.E.E. virus intraperitoneally. The results of the two experiments are presented in Fig. 8.

The difference between the titer of active Eastern strain of virus injected intraperitoneally into non-vaccinated control mice and that in vaccinated mice on the 4th day after the beginning of vaccination with the homologous strain was 100 to 1,000-fold. The titer of the same virus in mice vaccinated with the heterologous (Western) strain was ten fold less than in the non-vaccinated control mice. This may represent a small amount of non-specific resistance,

and, indeed, such resistance was found in vaccinated mice on the 2nd and 3rd days after beginning of vaccination, regardless of whether the vaccine was of the homologous or heterologous strain. However, the resistance induced by the 4th day as a result of vaccination with the homologous strain was significantly greater than that by the heterologous strain and was therefore of the nature of a specific immunity.

By the 8th day after the beginning of vaccination of mice 7 days old, a high degree of immunity to active virus of the Western strain (1,000,000 peritoneal units) had been induced by means of the homologous vaccine. This proved to be a highly specific immune response when compared with the lack of resistance of mice vaccinated with the heterologous strain. The degree of im-

4th day after beginning of vaccination of 10-days-old mice				8th day after beginning of vaccination of 7 days-old mice			
Dilution of active Eastern virus I.P.	Non- vaccinated litter mates	Vaccinated mice		Dilution of active Western virus I.P.	Non- vaccinated litter mates	Vaccinated mice	
		Western	Eastern			Eastern	Western
				10 ⁻¹			■■■■
				10 ⁻²			□□
				10 ⁻³			□□
10 ⁻⁴		■■■■■	■■■■□	10 ⁻⁴		■■■	□
10 ⁻⁵	■■■■■	■■■■■	■■■■□	10 ⁻⁵	■■■■	■■	
10 ⁻⁶	■■■■□	■■■■□	□□□□	10 ⁻⁶	■■■■□	■■■	
10 ⁻⁷	■■■■□	■■■□	■■■■□	10 ⁻⁷	■■■■□	■■	
10 ⁻⁸	■■■■□			10 ⁻⁸	□□□		

■=1 mouse died □=1 mouse survived

FIG 8 Specificity of immunity induced by vaccination with formalized W E E or E E E virus as tested by intraperitoneal injection of active virus

munity on the 4th and 8th days can hardly be compared since the intervals used and the ages of the animals differed.

These experiments proved that the resistance induced by vaccination with formalin-inactivated virus and tested by intraperitoneal injection of active virus was a true immunity specific for the Eastern or the Western strain of equine encephalomyelitis virus.

Infectivity of Blood of Mice after Intraperitoneal Injection of Active Virus

The susceptibility of young vaccinated mice to injection of active virus by cerebral and peripheral routes, compared with that of adult normal and young normal mice, has been studied. It was then of interest to find how much virus was recoverable from the blood of each of these groups after intraperitoneal injection of active virus, since virus introduced peritoneally reaches the central nervous system by way of the blood stream (8 a, d).

10^6 cerebrial units of active E E E virus were injected intraperitoneally into three groups of mice. One group consisted of normal 2 months-old mice, another, of normal 15-days-old mice and the third of 15-days-old mice which had been vaccinated, beginning at 7 days of age by four intraperitoneal injections of 0.1 cc. of formalin inactivated E E E virus on alternate days. The six mice in each group were bled from the tail, the blood samples of each group were collected in a test tube containing a drop of sterile solution of 1/500 heparin in saline. Bleedings were made at 2 hours, 24 hours, and 48 hours after the injection of active virus and at 6 days in the two surviving groups. 0.03 cc of each pool of blood, and dilutions in broth where indicated was injected intracerebrally into three mice. The fate of the injected mice as well as the infectivity of blood at intervals are presented in Fig 9.

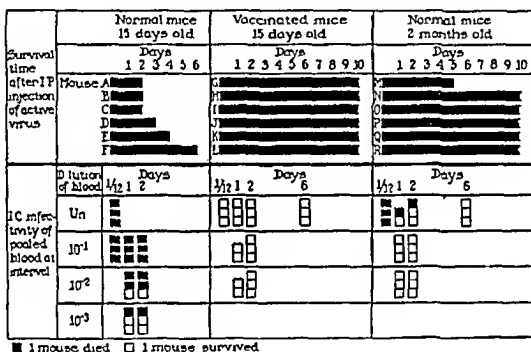


FIG 9 Effect of intraperitoneal injection of active E E E virus in mice and subsequent infectivity of the blood

Thus all six non vaccinated 15-days-old mice died within 6 days after injection of active virus, none of the six vaccinated 15-days-old mice died and one of the six normal 2 months-old mice died on the 5th day. This is consistent with previous experience that is young mice were susceptible to active virus by the intraperitoneal route whereas the majority of old mice were resistant furthermore young mice, as a result of vaccination became immune to the active virus given by this peripheral route.

Active virus was recoverable from the blood of the young normal group 2 hours after intraperitoneal injection at 24 and 48 hours, a 100 fold dilution of blood was fatal to two of three mice and a 1 000 fold dilution to one of three. In contrast to this no active virus was recovered from the blood of the young vaccinated mice at these intervals, nor at 6 days. Sufficient virus was present at 2 hours in the blood of the adult normal group to kill all of three mice injected intracerebrally, but only a minimal amount at 24 and 48 hours one of two and one of three injected mice re

spectively died. None was recoverable at 6 days. However, since one animal died on the 5th day, the recovery of a minimal amount of virus up to that time may have been due to virus in the blood of this individual rather than to a low concentration of virus in the blood of all.

Thus, after intraperitoneal injection of active virus in young normal mice, virus was demonstrable in 100- and 1,000-fold dilutions of blood for at least 2 days, all animals succumbed. In adult normal mice virus was recoverable in the blood at 2 hours, a minimal amount at 24 and 48 hours, and none at 6 days, the group as a whole was resistant to injection of active virus. Finally, as a result of vaccination, no virus appeared in the blood of young immunized animals, all of which survived. This is an example of another way in which young vaccinated animals in their response to active virus more closely resembled adult than young normal mice.

RÉSUMÉ AND DISCUSSION

The experiments reported deal with the influence of age on increasing capacity to elicit an immune response as correlated with decreasing susceptibility to peripheral injection of a neurotropic virus. The reaction of mice to Eastern equine encephalomyelitis virus was studied.

After intraperitoneal injection of active virus into mice of various age groups, a larger proportion survived with greater age, and of those which succumbed, the greater the age, the longer the incubation period. In the serum of surviving mice of the various age groups no difference in rate of development of serum-neutralizing antibodies was found. It should be recalled that survivors were not available in the younger groups on the 5th day when antibodies were first clearly demonstrable in the serum of adults. Since it was inherently impossible to obtain representative groups of each age at intervals after injection with active virus, immune response to formalin-inactivated virus was studied. Young mice, after vaccination with inactivated virus, developed peritoneal resistance in the absence of cerebral resistance at a time when their serum showed little or no demonstrable antibody (14). The degree of peritoneal resistance induced in young mice increased with very small increments of age. This increase in resistance was paralleled by increase in serum-neutralizing antibodies. Thus the amount of serum-neutralizing antibody was found to be an index of peritoneal resistance after vaccination. It was, however, an insensitive indicator, since the neutralizing capacity of a sample of serum was consistently lower than the peritoneal resistance of the animal, as shown by comparing the amount of virus neutralized with the amount resisted. The peritoneal resistance induced was found not to be local, *i e*, confined to the peritoneum, but rather a systemic immunity of non-nervous tissue, specific for the Eastern strain of equine encephalomyelitis virus.

An explanation offered for an acquired resistance of growing animals to peripheral injection of certain neurotropic viruses in the absence of humoral antibodies and of previous exposure to infection was the development of barriers with age which served to arrest the progression of virus (7 b). Since no anatomical changes were observed to account for these barriers, they were considered hypothetical barriers, or silent areas beyond which the virus could not pass. In the present experiments an attempt was made to find to what extent the greater rate of immune response of older animals might account for their insusceptibility to virus given peripherally. A parallel was drawn between the reaction of young vaccinated mice and adult normal mice to peripheral injection of active virus. Young vaccinated mice simulated adults in that although fully susceptible to virus given by the intracerebral route, they resisted large doses of virus by the intraperitoneal route. It should be remembered, however, that their serum contained minimal amounts of neutralizing antibody.

Another comparison between adult mice, and young mice vaccinated and non vaccinated, was based on the recovery of virus from their blood after intraperitoneal introduction of active virus. Since virus is known to invade the central nervous system by being deposited from the blood onto the nasal mucosa, it was of interest that a minimal amount of virus was found in the blood of adults, none in that of young vaccinated, and a large amount in that of young non vaccinated mice. The lack of large amounts of circulating virus in the blood of adult mice would explain why invasion of the central nervous system in most individuals failed to take place, although it was fully susceptible at the time. The possibility must not be overlooked that failure to recover large amounts of virus may have been due in part to an insusceptibility of non nervous tissues of the adult. Although immune response is set in motion at the time of injection of active virus (14), at just what point it becomes effective is difficult to define. It has been shown that 10-days-old mice had developed a specific immunity to active virus injected intraperitoneally by the 4th day after beginning of vaccination. Older animals, capable of a more rapid immune response, would be expected to have developed an effective immunity within that time.

Although the central nervous system of old and young mice was found equally susceptible to Eastern equine encephalomyelitis virus, with increasing age there was a decreasing susceptibility to virus introduced by the peritoneal route. Evidence has been presented to show to what extent the more rapid and greater immune response of older animals might have influenced the outcome of such peripheral injection.

No generalization is intended that young animals immunized by means of other viruses would always present a greater resistance of non nervous tissue compared with that of the central nervous system. This relationship has

been found to obtain for Eastern equine encephalomyelitis virus, a virus to which nervous tissue is the most susceptible. The relative resistance of the tissues of the host to each virus would have to be studied. For rabies virus, another neurotropic virus, there is already evidence (15) of decreasing susceptibility with age of mice, and furthermore mice are more readily immunized with increasing age. As with Eastern equine encephalomyelitis virus, immunity is more readily induced to peripheral than to cerebral injection of virus.

Other factors which influence degree of immune response may be mentioned. That hormonal factors may influence immunizability has been brought out by Hodes (18*a*) who found a reduction in the capacity of mice to elicit an immune response to St. Louis encephalitis virus during pregnancy. Sabin and Duffy (18*b*) have demonstrated that diet may affect the age at which susceptibility of mice to peripheral injection of the neurotropic virus of vesicular stomatitis changes. It would be of interest to know whether the difference in diet affects the degree to which animals may elicit an immune response.

Although relatively few cases of infection of man with the virus of equine encephalomyelitis have as yet occurred, it is of interest to consider what bearing the experiments reported may have on the human disease. The age difference in the milder infection by the Western strain is not marked (19). In the Massachusetts epidemic (2) caused by the Eastern strain, children were predominantly affected. The majority of cases terminated in death, and in those which survived permanent cerebral injury occurred (20*a*). Although Fothergill (20*b*) was unable to find antibodies in the sera of adult contacts available for study, he stated that his negative findings did not exclude the possibility of inapparent infection in adults. Antibodies to the Eastern strain have not only been demonstrated in the serum of recovered cases but also in the serum of apparently healthy individuals associated with laboratory work on the virus (21). In summary, infection due to equine encephalomyelitis virus, Eastern strain, has proved fatal to children, or has left permanent cerebral injury, whereas in adults, inapparent infections have occurred as evidenced by serum-neutralizing antibodies in the absence of symptoms.

SUMMARY

The experiments described in this paper were carried out with the Rockefeller Institute strain of albino mice and with the Eastern strain of the virus of equine encephalomyelitis.

- 1 The observation was confirmed that with increasing age of mice there occurred a decrease in susceptibility to intraperitoneal injection of active virus, also, the length of incubation period of those which succumbed increased with age.

- 2 The mice of various age groups which survived an intraperitoneal injection of active virus were indistinguishable in their antibody response.

3 Young mice, vaccinated with formalin inactivated virus when 2, 5, and 7 days old, gave an immune response to such a degree that they showed (a) measurable peritoneal immunity which increased with small increments of age, (b) no cerebral resistance, and (c) detectable amounts of neutralizing antibody in their sera which paralleled, though at a considerably lower level, their peritoneal resistance

4 The peritoneal resistance induced as a result of vaccination was shown to be not local, but a general, systemic immunity, specific for the Eastern strain. Such a peritoneal resistance was demonstrable by the 4th day after beginning of vaccination of 10-days old mice

5 After intraperitoneal injection of active virus, large amounts of virus were recoverable from the blood of non vaccinated young mice, none was found in the blood of vaccinated young mice, a minimal amount was detectable in the blood of non vaccinated adult mice

6 The bearing of age on the degree of immune response of which mice are capable and on their susceptibility to the virus has been discussed

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CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

V A FLAVIN ASSOCIATED WITH THE PURIFIED VIRUS

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Elementary bodies of vaccinia exhibit uniformity in their chemical constituents and biological activity (1-3) Chemical, physical, and immunological studies have indicated a degree of complexity for this animal virus which is not shared by certain members of the group of crystallizable plant viruses The view, therefore, that respiratory catalysts and certain specific growth substances, which are known to play an important rôle in bacterial metabolism, may function in the organization of elementary bodies of vaccinia is not untenable

Modern researches by Warburg, Keilin, Meyerhof, and others, in the field of cellular catalysis, have led to the view that, although many substances may participate in a cellular reaction, certain well defined organic catalysts, often functioning as prosthetic groups of enzymes, take up key positions along the metabolic chain which begins with the oxidation of substrate and ends in many instances in the reduction of molecular oxygen Of these catalysts, the flavins, the phosphopyridine nucleotides, and the cytochromes are important and have been well studied Search for a cytochrome component of vaccine virus was unsuccessful (3) However, a copper constituent in constant amounts in purified elementary bodies of vaccinia has been found, indicating that a highly organized virus, such as vaccinia, may possess substances which function catalytically in biological oxidations

Riboflavin is among the more important oxidative catalysts known to play a rôle in cellular metabolism This substance when linked to phosphate and adenylic acid forms a nucleotide, and in this combination functions as the prosthetic group of a number of well defined enzymes concerned with the oxidation of coenzymes I and II, with the deamination of unnatural amino acids, and with the oxidation of xanthine and aldehydes (4) A new member of this group of flavins which is concerned with the oxidation of reduced cytochrome c has been recently reported by Haas, Horecker, and Hogness (5) In view of the ubiquity of flavin containing enzymes in animal, plant, and bacterial cells a search for components of this system in the elementary body

of vaccinia has been made. The results of the work are reported at this time.

EXPERIMENTAL

In the course of extraction of nucleic acid from elementary bodies of vaccinia it was observed that alkaline solutions of the purified virus gave a marked fluorescence in the presence of ultraviolet light. Increased alkalinization caused an irreversible destruction of the fluorescent property. Moreover, it was noted that suspensions of elementary bodies of vaccinia tended to lose this fluorescence to some extent on standing for prolonged periods of time in strong light. The fluorescence was partially regained, however, when the light-exposed virus was placed in the dark for several hours. Flavins are characterized in part by their instability in the presence of strong light (6). This reaction, known as photolysis, is greatly accelerated in an alkaline medium. It, therefore, occurred to us that the behavior of the virus in ultraviolet light could be explained in part, at least, if a flavin constituent were demonstrated.

Chromatographic Separation of Fluorescent Material from Virus

It was possible by treating vaccine virus with 50 per cent alcohol in a buffer at pH 4, to dissociate from the virus a substance which carried a major part of the total fluorescence. Moreover, it was possible by means of chromatographic methods to concentrate the material in a thin ring in an absorption tower of aluminum oxide from which it was subsequently eluted with sodium bicarbonate and methanol.

Construction and Use of Chromatographic Tower—50 mg of elementary bodies of vaccinia were suspended in 10 cc of water, and 0.1 N hydrochloric acid added until the pH of the suspension was 4. The volume was brought to 15 cc with distilled water, and 15 cc of absolute alcohol were added slowly with vigorous stirring. The alcohol-elementary body mixture was heated for 5 minutes on a boiling water bath and filtered (Whatman No. 42 filter paper). The clear filtrate, examined with ultraviolet light, exhibited a marked bluish green fluorescence. An absorption column was constructed, which consisted of soft glass tubing, 1.5 cm in diameter and 35 cm in length, with a constriction at one end. A small portion of glass wool was packed tightly against this constriction and Brockmann anhydrous aluminum oxide (Al_2O_3) was packed firmly against the glass wool until the column was filled to within 5 cm of the top. Another portion of glass wool was packed at the top against the aluminum oxide. The tower was clamped to a stand and suction was applied at the constricted end, after which the filtrate from the heated elementary bodies was added slowly from above. The speed of flow of the filtrate through the aluminum column was controlled by the amount of suction applied. About 15 minutes was required for the passage of each 10 cc of filtrate. It was necessary to pack the tower tightly enough for the fluid to descend slowly, wetting the aluminum oxide in an advancing sharp plane, otherwise

irregular channels appeared in the aluminum oxide and sharp separation of the zone was not obtained

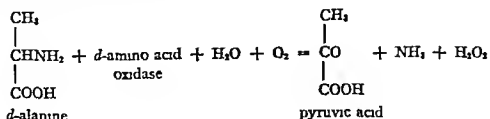
Development of the Chromatogram—It was possible by frequent examination of the tower with a mercury arc light to note the changing position of the zone of fluorescence. 10 cc portions of 4 normal hydrochloric acid alcohol mixture were added until the ring had advanced 5 to 10 cm below the top of the aluminum oxide column. By means of a file the absorbing column was broken cleanly just above the fluorescent ring and the aluminum oxide carrying the zone of fluorescent material was lifted out by means of a spatula and placed in a small beaker. 10 cc. of methanol sodium bicarbonate solution were added to the aluminum oxide absorption complex and the mixture shaken vigorously and filtered. The filtrate containing the fluorescent material was neutralized with 0.1 N hydrochloric acid and concentrated *in vacuo* to 5 cc. The material eluted from the ring of aluminum oxide was pale yellow in color and gave an intense bluish yellow fluorescence in the presence of ultraviolet light.

Absorption Spectrum of the Fluorescent Material—Attempts to identify the fluorescent substance by a study of the absorption spectrum failed. There was considerable end absorption in the ultraviolet, indicating the presence of extraneous material. Although marked fluorescence of the eluate was observed, there was insufficient concentration to render identification by absorption spectra possible, either in the visible or ultraviolet region of the spectrum. We accordingly turned to other methods for its identification.

Identification of Flavin

If the material responsible for any part of the fluorescence of the virus could be attributed to flavin, it was most likely bound in a flavoprotein combination, since free flavin would almost certainly have been washed away in the extensive process of virus purification.

In 1935 Krehs (7) described a technique for the estimation of flavin adenine-dinucleotide making use of the protein of the *d* amino acid oxidase. *d* Amino acid oxidase is a conjugated enzyme, with flavin adenine-dinucleotide as its prosthetic group. It is readily prepared in large quantities from pig kidney. The intact enzyme specifically catalyzes the conversion of the unnatural amino acids to the corresponding alpha keto acids, ammonia and hydrogen peroxide. So far as it is known, all *d*-amino acids undergo oxidative deamination in the presence of *d* amino acid oxidase, although great variation in rate of oxidation exists among the individual members of the *d* amino acid series (7). For most purposes *d* alanine has been used as a substrate because of its ready availability and the rapidity of its deamination. The over all reaction may be written thus:



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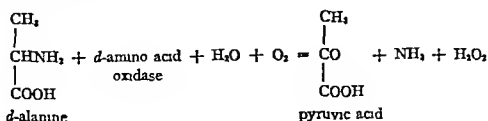
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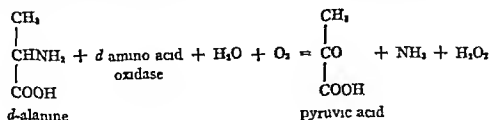
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Under carefully controlled conditions, worked out by Warburg and Christian (9), the above reaction can be made quantitative for the determination of flavin-adenine-dinucleotide. This is accomplished by dissociating the flavin of *d*-amino acid oxidase by lowering the pH to 4, and precipitating the protein portion of the enzyme from solution with ammonium sulfate. The protein alone is inactive, but becomes very active in the conversion of *d*-alanine to pyruvic acid when mixed with solutions containing flavin-adenine-dinucleotide. The oxygen uptake in the Warburg manometer in the presence of the specific protein and *d*-alanine is proportional to the flavin-adenine-dinucleotide in the test solution. According to Warburg and Christian (9) the reaction between the *d*-amino acid oxidase and *d*-amino acid, *i.e.*, the rate of oxygen uptake in the presence of an excess of *d*-amino acid, may be expressed by the equation $\text{Rate of } O_2 \text{ uptake} = k (\text{flavin}) (\text{protein})$.

The equation indicates that the rate of oxygen uptake will be increased in three ways (1) by an increase in the flavin-adenine-dinucleotide component, (2) by an increase in the quantity of the specific protein, and (3) by an increase in both these constituents. Warburg and Christian (9) and others (10) have taken advantage of this equation to evolve a technique for the estimation of flavin-adenine-dinucleotide by determination of the constant of the protein in the presence of a known amount of pure flavin-adenine-dinucleotide. They found the relation between the activity of a fixed quantity of protein and *d*-amino acid, with varying amounts of flavin-adenine-dinucleotide, to be expressed by the equation $k = c \frac{V_0 - V}{V}$ where *c* is the concentra-

tion of flavin-adenine-dinucleotide which will produce rate *V*, and *V*₀ is the rate in the presence of an excess of flavin-adenine dinucleotide. When $V = 0.5 V_0$, *k* becomes equal to *c*, and can be expressed in micrograms of flavin-adenine dinucleotide present in the test substance. By comparison of the rate produced by known amounts of flavin-adenine-dinucleotide with the rate produced by an unknown substance in the presence of a given quantity of the protein of the *d*-amino acid oxidase, the concentration of flavin-adenine dinucleotide in the unknown substance can be determined.

Preparation of *d*-Amino Acid Oxidase—*d*-Amino acid oxidase from pig kidney was prepared according to the method of Warburg and Christian (9), after which the specific protein was recovered free of prosthetic group by precipitation with ammonium sulfate.

To a fine mince of fresh pig-kidney cortex, 5 volumes of chilled acetone were added. The mixture was stirred vigorously for 5 minutes, after which it was filtered through a Buchner funnel. The precipitate was dried *in vacuo* at 4°C, then powdered, and kept in the cold as a source of starting material for the preparation of the specific protein.

To prepare the protein portion of the enzyme, 200 cc of water were added to 10 gm of the dried kidney powder. The material was next centrifuged and the sediment discarded. To 200 cc of the clear supernatant solution at 0°C were added 13.3 cc. of 1 molar acetate buffer, pH 3.8. The precipitate was collected by centrifugation and discarded. To the supernatant material kept at 0°C was added ½ volume of saturated ammonium solution. The precipitate which formed slowly was collected by centrifugation in the cold, dissolved with 5 cc of 0.1 molar sodium pyrophosphate buffer, pH 8.3, and made up to 100 cc with water. To this dilute solution were added

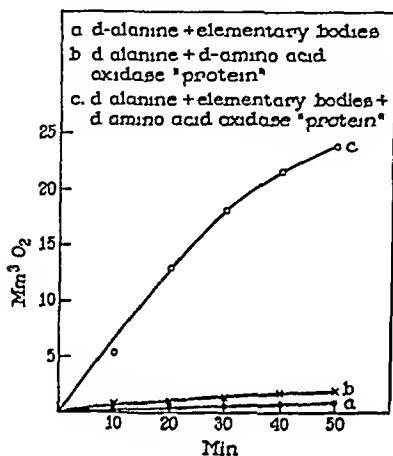
35 cc. of saturated ammonium sulfate, and while being shaken 40 cc of 0.1 normal hydrochloric acid were added slowly. At this time the pH of the solution was 2.8, and the ammonium sulfate concentration was about 0.2 molar. The precipitate was collected by centrifugation, dissolved in 5 cc of 0.1 molar sodium pyrophosphate, and made up to 100 cc with water. Precipitation with ammonium sulfate and hydrochloric acid was repeated as before and the precipitate taken up in 2.5 cc of 0.1 molar sodium pyrophosphate and made up to 25 cc with water. The solution was clarified by centrifugation, after which it was frozen and dried *in vacuo*.

Qualitative Determination of Flavin Adenine Dinucleotide in Elementary Bodies—The presence of flavin adenine-dinucleotide in the elementary bodies was demonstrated in the following manner:

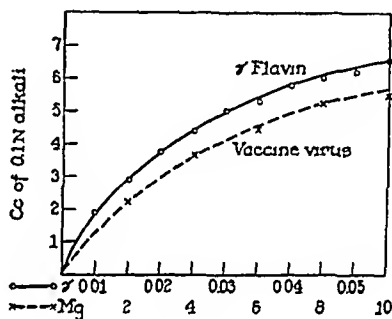
11 mg of freshly prepared elementary bodies of vaccinia were suspended in 1.1 cc. of water and heated for 10 minutes at 80°C. Fluid lost by evaporation was replaced, and 1.0 cc of the suspension containing 10 mg of virus was placed in the bottom of a conical Warburg flask. The central well of the flask contained a small roll of filter paper and 0.2 cc 5 N NaOH. 2 mg of specific *D*-amino acid oxidase 'protein' in 0.5 cc. of 0.1 molar sodium pyrophosphate buffer pH 8.3, were added to the elementary body suspension and thoroughly mixed by gentle rotation. 1 mg of *D*-alanine in 0.5 cc. of the pyrophosphate buffer was added to the side arm. The flask was attached to the manometer and the manometer flask system equilibrated with pure oxygen. The manometers were then shaken in the water bath until equilibrium had been achieved and the *D*-alanine in the side arm was tilted into the reaction flask. The manometers were shaken and oxygen consumption observed at 10 minute intervals for a period of 2 hours. Control flasks, containing specific 'protein' and *D*-alanine, elementary bodies and *D*-alanine, and specific 'protein' and elementary bodies were set up at the same time.

No reaction was observed in the control flasks. A steady oxygen uptake in the flask containing elementary bodies, "specific protein," and *D*-alanine of about 4 to 5 mm of oxygen per 10 minute interval was noted consistently. Oxygen consumption was likewise shown on successive lots of elementary bodies prepared in the same manner. A graph for a typical reaction rate is given in Text fig. 1. When unheated elementary bodies were used in the set up described above, oxygen utilization was likewise observed, but at a significantly lower rate. The increased rate of oxygen uptake observed in the case of elementary bodies heated to 80°C can be explained in part by the fact that for maximum activity it is necessary first to release flavin adenine dinucleotide by denaturation of the virus, and in part by the fact that heat inactivates the trace of catalase which, as Macfarlane and Salaman have shown, is associated with virus preparations (8). This catalase, which we have also found in our elementary body preparations, unless inactivated by heat or cyanide, served to release oxygen from the hydrogen peroxide which is formed in the reaction mixture thereby lowering the apparent oxygen uptake in the Warburg manometer.

Quantitative Determination of Flavin-Adenine-Dinucleotide in the Virus by Enzymatic Reaction—An attempt was made to estimate the amount of flavin-adenine-dinucleotide in elementary bodies by comparison of the rate of reaction of the protein of *d*-amino acid oxidase and heated virus, with that of the protein and flavin-adenine-dinucleotide prepared from yeast. For this purpose it was necessary to prepare pure flavin-adenine-dinucleotide according to the method of Warburg and Christian (9)



TEXT-FIG 1



TEXT-FIG 2

TEXT-FIG 1 Oxidation of *d*-alanine by elementary bodies of vaccinia in the presence of the "protein" of *d*-amino acid oxidase

TEXT-FIG 2 Growth response of *Lactobacillus casei* E in a basal medium to pure riboflavin and to elementary bodies of vaccinia

10 kilos of fresh yeast were mixed with 20 liters of hot water and held at 75–80°C for 10 minutes with stirring, after which the residue was removed by centrifugation. To the supernatant fluid were added 11 kilos of ammonium sulfate and the dinucleotide extracted from the precipitated material by three successive extractions with 2.5 liters of phenol (U.S.P., 88 per cent). The phenol mixture was next extracted successively with a total of 6 liters of ethyl ether and 1.5 liters of water. The aqueous solution of dinucleotide, freed from ether by distillation *in vacuo*, was made acid to Congo red with 140 cc of 2 normal nitric acid and the dinucleotide precipitated with 20 cc of 30 per cent silver nitrate. The silver precipitate was next collected by centrifugation and washed with 100 cc of water, suspended in 50 cc of water, and decomposed with hydrogen sulfide. The dinucleotide was extracted from the silver sulfide by washing the precipitate eight times with 25 cc of molar/100 barium acetate. This solution, containing the barium salt of the dinucleotide, was made acid with 6 cc of normal sulfuric acid, and the barium sulfate precipitate centrifuged and washed with water. To the aqueous extract were next added 150 gm of ammonium sulfate,

and the resulting mixture extracted with 15 cc of paracresol. The paracresol solution containing the dinucleotide, was washed many times with 0.2 normal solution of sulfuric acid containing 50 per cent, by weight of ammonium sulfate. 500 cc of ethyl ether were added to the paracresol solution and the mixture shaken with water. The aqueous layer, recovered by means of a separatory funnel, was made acid to litmus and sufficient 2 molar barium acetate added to remove the sulfuric acid as barium sulfate. A phosphorus analysis on the aqueous material was performed, and for each gram atom of phosphorus found one mole of barium acetate was added. The mixture was concentrated *in vacuo* to about 10 cc. and 2 moles of ammonium sulfate added for each gram atom of phosphorus, after which the mixture was dried *in vacuo* and the ground dried residue extracted several times with 95 per cent alcohol. The alcohol was discarded, the residue extracted at 40°C with 2.5 cc of water, and the aqueous extract discarded. The brown residue was then dissolved in 10 cc. of water at 60°C. On cooling to room temperature crystals of the barium salt of flavin adenine-dinucleotide were precipitated. These were washed with absolute alcohol and dried *in vacuo* at 0°C.

The quantitative determinations of the amount of flavin-adenine-dinucleotide in vaccine virus were conducted in the following manner:

Flavin adenine-dinucleotide in amounts less than 0.5 microgram was dissolved in 0.5 cc. of water and placed in the bottom of a Warburg respiration flask. 2 mg of specific *d* amino acid oxidase protein in 0.5 cc of 0.1 molar sodium pyrophosphate buffer, pH 8.3, were added and mixed thoroughly by gentle rotation. 1 mg of *d* alanine in 0.5 cc. of pyrophosphate buffer was added to the side arm. The flask was attached to the manometer, and the system equilibrated with pure oxygen. The manometer cock was closed and when temperature equilibrium had been achieved in the water bath at 38°C the *d* alanine in the side arm was tilted into the reaction flask and the manometer observed at 10 minute intervals for oxygen uptake. Control flasks containing the specific protein and *d* alanine, and flavin adenine-dinucleotide and protein were set up at the same time. In another set of flasks, heated elementary bodies, as a source of flavin adenine-dinucleotide and appropriate controls were set up in the manner previously described, using a 2 mg aliquot of the specific protein for each 10 mg lot of heated elementary bodies.

The results are given in Table I. 0.0002 and 0.0001 mg of flavin adenine-dinucleotide yielded rates respectively slightly higher and slightly lower than the rate given by 10 mg of elementary bodies of vaccinia. Although the identification of flavin adenine-dinucleotide by this technique was possible, absolute quantitative results were hard to secure for three reasons: (1) it is difficult to know by any standards thus far brought forward when one has obtained a pure flavin adenine-dinucleotide from yeast, (2) it is a laborious task to prepare and keep standardized, active solutions of the specific protein from *d* amino acid oxidase, since the material becomes irreversibly inactivated upon standing in solution and (3) it requires several determinations on each dilution of material to locate accurately the point in the equation where $V = 0.5 V$.

Moreover, when amounts of virus greater than 10 mg were used for the determination of flavin-adenine-dinucleotide by this procedure an unexplained inhibition of the reaction seemed to occur so that total activity was actually less than that observed with smaller quantities of virus. When amounts of virus smaller than 10 mg were used, the rates were often too low for accurate manometric measurement. Under the best of conditions the quantitative results obtained by this technique can be regarded only as an approximation. For these reasons we turned to yet another procedure for the quantitative determination of flavin in our virus material.

Quantitative Determination of Riboflavin in Elementary Bodies of Vaccinia by Microbiological Assay—In 1939, Snell and Strong (11) described a relatively simple method for the quantitative determination of riboflavin. The method is based on the requirements of *Lactobacillus casei* E for this substance as a growth factor. To a basal medium, in which all the growth requirements of

TABLE I

Oxidative Deamination of d Alanine by the "Protein" of d Amino Acid Oxidase Together with Elementary Bodies of Vaccinia and with Flavin-Adenine-Dinucleotide Prepared from Yeast

Test material	Amount	Protein ^a of d amino acid oxidase	O ₂ uptake
	mg	mg	mm ³ per hr
Elementary bodies of vaccinia	10.0	2.0	22.0
Flavin-adenine dinucleotide	0.0001	2.0	17.9
" " "	0.0002	2.0	24.2

this bacillus except riboflavin are present in excess, is added a finely divided suspension of the material to be assayed for riboflavin. The mixture of medium and substance to be tested is then autoclaved, and when cool inoculated with a fresh, washed culture of *Lactobacillus casei* E. The culture is allowed to incubate 72 hours, or until no further growth of the bacilli occurs. A set of control tubes containing the basal medium to which known increments of crystalline riboflavin have been added is prepared at the same time, autoclaved, inoculated with *Lactobacillus casei* E, and incubated for the same period. Since riboflavin in the test material is the factor limiting growth of the organism, both the number of organisms, as measured by turbidity, and the amount of lactic acid produced, as measured titrimetrically, bear a relationship to the quantity of riboflavin present. By this technique purified vaccine virus has been shown to contain riboflavin, and the amount has been expressed in micrograms of riboflavin per unit weight of the dry virus.

A set of culture tubes containing 10 cc of basal medium prepared according to the directions given by Snell and Strong (11) were set up in duplicate and increments of riboflavin added in amounts of 0.0, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, and 0.40 micro-

grams A set of tubes with the basal medium containing freshly prepared vaccine virus, in amounts of 2.0, 4.0, 6.0, 8.0, and 10.0 mg were set up at the same time Both the standard medium containing increments of riboflavin and the medium containing vaccine virus, in lieu of riboflavin were autoclaved at 20 pounds for 15 minutes, cooled to room temperature, and inoculated with 0.05 cc. of a 24 hour culture of *Lactobacillus casei* E This culture had been grown on a medium containing 0.1 microgram of added riboflavin, washed twice with phosphate buffer, and resuspended in 20 cc. of sterile physiological saline The tubes containing the standard and those with the virus material being tested were then incubated at 38°C for 72 hours At the end of this time the growth turbidity was measured by means of a photocell colorimeter of the Evelyn type, and the lactic acid was titrated with 0.1 normal sodium hydroxide to a brom thymol blue end point In general, the results of duplicate samples obtained by titration agreed more closely than results obtained

TABLE II

Flavin Content of Purified Elementary Bodies of Vaccinia as Determined by Assay with Lactobacillus Casei E

Lot	Virus taken for assay	Flavin per 100 gm of virus
	mg	mg
1	2	1.5
2	2	1.5
3	2	1.4
4	2	1.5
5	2	1.4
6	2	1.5

by turbidity measurements, and were finally used exclusively as a measure of growth and consequent metabolic activity of the culture

Determinations by means of *Lactobacillus casei* E done on 10 lots of virus reveal, from lot to lot, a fairly constant content of riboflavin, ranging from 1.1 and 1.5 mg per 100 gm of virus The results obtained by microbiological assay are given in Table II A graph of the growth response of *Lactobacillus casei* E to pure riboflavin and to elementary bodies of vaccinia is given in Text fig 2

Separation of Flavin Constituent from Elementary Bodies of Vaccinia

All attempts to separate the flavin from purified elementary bodies of vaccinia by procedures which in themselves do not inactivate the virus have thus far failed, namely, repeated washing with buffers ranging from pH 6 to pH 8, ultrafiltration by means of the Coolidge apparatus (12) or electrodialysis

Attempt to Separate Flavin from Virus by Repeated Washing in Dilute Buffers—On the assumption that the flavin adenine-dinucleotide present in purified virus might have been adsorbed from the extraneous animal material

during the process of preparation of the virus, we set about to see if it could be eluted by methods which are of recognized value in the separation of adsorbed substances. For flavin constituents, certain of the factors influencing adsorption and elution are known. Among these is the factor of pH. In general, adsorption of flavin is favored at a low pH level and elution favored within higher pH ranges.

To 20 mg of purified elementary bodies of vaccinia were added 20 cc of 0.025 molar citrate buffer of pH 4. The virus was removed by means of centrifugation and resuspended and washed twice in distilled water. The virus was finally collected by centrifugation, dried at 0°C *in vacuo*, and assayed for flavin by means of the microbiological method previously described. The experiment was repeated, but citrate buffers of pH 5 and 6.5, and 0.075 molar phosphate buffers at pH 7, 7.5, and 8.0 were used. No significant loss of flavin could be detected following these repeated washings with buffers of varying pH.

Attempt to Separate Flavin Constituent by Ultrafiltration—For ultrafiltration a simple device described by Coolidge (12) was used. By this method Coolidge was able to show that bilirubin was attached to the albumin fraction of human serum and could not be removed by ultrafiltration.

10 mg of purified virus were suspended in 10 cc of dilute buffer at pH 7.2 and placed in a cellophane bag. The end was knotted, and the bag placed vertically in a small tube of the Coolidge type, supported by a porous plate which rested in turn on a partial constriction at the middle of the tube. The assembled tube was centrifuged at 2000 R P M for 1 hour. After this time about 5 cc of ultrafiltrate were obtained. The ultrafiltrate, tested microbiologically, did not contain riboflavin. Moreover, no fluorescence of this material was observed in ultraviolet light.

Attempt to Separate Flavin Constituent by Electrodialysis—An apparatus and procedure for electrodialysis which does not inactivate vaccine virus has been described previously (3). By this technique it is possible to control the pH at any desired level by means of dilute buffer which flows steadily through the protecting cells placed between the virus and electrodes.

10 mg of virus were suspended in dilute buffer in the center cup of a 5-compartment electrodialysis cell, and dialyzed with a potential of 110 volts and a current of 50 milliamperes for 48 hours at 20°C. Aseptic precautions were insured by autoclaving the cell before use and the cautious introduction of bacteria-free virus. That no bacterial contamination or growth occurred was shown by cultures made on blood agar plates at frequent intervals during the course of the dialysis.

Electrodialysis produced no significant drop in the flavin content of the virus over that noted in a control lot of virus kept at 20°C and at the same pH.

DISCUSSION

All attempts to separate the flavin component from elementary bodies of vaccinia by means which did not result in inactivation and denaturation of the virus failed, namely, repeated washing with buffers ranging from pH 6 to 8, ultrafiltration by means of the Coolidge apparatus, and electro dialysis. The only successful means thus far found to remove the flavin constituent has been to lower the pH to 4 and precipitate the virus from suspension by means of heat and alcohol, methods which are known to dissociate flavin adenine dinucleotide from its protein component.

With respect to the concentration of riboflavin in vaccine virus, it is interesting to note that it compares favorably with that found in muscle, but it is significantly below that in yeast which is an exceptionally rich source of riboflavin. The published values also show a greater concentration of riboflavin in bacteria than that found in vaccine virus, although a great range of variability in microorganisms is known to occur.

The demonstration that elementary bodies of vaccinia contain constant amounts of flavin adenine-dinucleotide is in itself an interesting fact, but proof that this substance is an integral part of the elementary body and that it functions in the metabolism of the virus obviously constitutes a much larger problem. It would be a most attractive hypothesis to assume that its presence there permits the elementary body to divert a certain portion of the energy derived from oxidative reactions, begun in the host cell, through its own flavin oxidation reduction system, thereby enabling it to live as an obligate parasite. So far, no direct evidence can be offered for this point of view, except that the presence of flavin adenine-dinucleotide constituent, if upon further work it is definitely demonstrated to be an active component of the virus, would indicate that the virus possesses in part a metabolic system akin to that of its host cell.

CONCLUSION

Suspensions of purified elementary bodies of vaccinia exhibit fluorescence in the presence of ultraviolet light. This fluorescent constituent can be separated by chromatographic methods provided the virus is first denatured by acid and heat. By means of the specific protein of *D* amino acid oxidase it has been possible to identify the flavin constituent as flavin adenine dinucleotide and show that it can participate in the oxidative deamination of *D* alanine. By means of microbiological assay the flavin component has been quantitatively measured and shown to compare favorably in concentration with that observed in animal cells and in some bacteria, its concentration in virus is lower than that observed in yeast. The demonstration that it exists as an integral portion of the virus is not conclusive. So far, however, it has been

separated from the elementary bodies only by means which in themselves inactivate the virus

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SECOND ATTACKS OF POLIOMYELITIS

AN EXPERIMENTAL STUDY*

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PLATES 7 AND 8

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It is generally conceded that a relatively substantial and lasting immunity to poliomyelitis is conferred through invasion of the nervous system by active virus, nevertheless, well authenticated second attacks of the disease do occur both in man and in experimental animals (1-9). This puzzling contradiction, which has led some to a denial of the existence of active immunity (1), arises in part at least from a lack of precise knowledge regarding the mechanisms of immunity production in the disease. For a solution of the problem one must have recourse to the experimental animal. It must be recognized at the outset that many strains of poliomyelitis virus do not produce consistent immunity against others (2, 4-9) that an animal is resistant to reinoculation by the same strain of virus is also accepted by most workers (7-11, *contra* 3). At the same time this substantial type of immunity which weathers large testing doses is thought to be a local one, residing in the central nervous system. There is a possibility, however, that such an immunity of the nervous system may be further restricted to include only portions of it. The following experiments and discussion will show that this is actually the case, which means that limited reaches only of the nervous system may be refractory to one strain of virus at a given time.

In the course of a series of investigations dealing with the influence of fiber tracts upon the propagation of poliomyelitis virus within the central nervous system of the monkey, it became apparent that although certain neurone systems were unquestionably highly susceptible to virus, nevertheless they did not always suffer visible signs of invasion (12). These differences in the total distribution of virus could be correlated with the portal of entry, and it was apparent that there were definite limits to the spread of virus even during a severe paralytic attack of poliomyelitis. Additional observations on this point are the subject of the present paper since the question immediately arose whether one invasion of the nervous system immunized it as a whole, or whether some of those portions which were susceptible but did not show lesions might

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still remain susceptible. This concept rapidly reduces to the proposition of whether a single attack of poliomyelitis automatically closes all portals of entry.

In order to establish the thesis that one attack of poliomyelitis does not immunize the entire nervous system it is necessary to produce paralytic poliomyelitis by one portal of entry and then subsequently attempt reinfection by a new portal which has not been involved in the first spread of the disease. Experiments designed to elucidate such a point are subject to certain limitations imposed by the fact that the host animal, the *rhesus* monkey, has only one "natural" portal. In this animal (contrast the chimpanzee (13)) intact skin and mucous membrane furnish an effective barrier to the entrance of virus at every point except the olfactory area, which may be regarded as the portal of choice for experiments dealing with immunity. Nevertheless when the barrier of the non-olfactory epithelium is broken and virus is brought into contact with any considerable part of the nervous system, infection regularly ensues. Once virus has gained access to the neuraxis there is no reason to doubt that it is disseminated through it with the same readiness as though it had been introduced through the olfactory portal. This being the case, it is possible to consider various portions of the nervous system as legitimate portals of entry which can be employed to induce an initial attack of paralytic poliomyelitis.

In order to control the second or testing inoculation it is necessary to show beyond all reasonable doubt that virus has not been present in the regions of the central nervous system which are to be exposed at this time. This necessitates the careful examination of the nervous tissues in many cases. Since the olfactory portal was to be used in most instances for the testing inoculation, the olfactory bulbs were examined in forty cases in which routes other than the olfactory were employed for the production of paralytic poliomyelitis. The ciliary ganglia were also studied in seven cases paralysed after intranasal inoculation since the intraocular route was also to be used for testing immunity in intranasal convalescent animals. All the tissues were examined in serial sections 10 to 20 μ in thickness stained with gallocyanin after the method of Emarson (14). Normality was determined by the presence or absence of lesions—a method of assay which in respect to a susceptible tissue like the olfactory bulb we have come to regard as more sensitive than subinoculation. The first group in Table I is composed of thirteen cases of severe paralysis induced by inoculations into various regions of the cerebral cortex, both motor (areas 4, 6, and 8), and sensory (area 5). On examination of the olfactory bulbs it was not uncommon to find a slight meningitis at the pial surface which was consistent with the presence of a general meningitis, but no true lesions were present in the bulbs except in one questionable case where a large inoculum was placed into one frontal pole. It seems very likely that this single positive case represented a direct contamination of the olfactory bulbs. Fur-

thermore in each of two cases following intracerebral inoculation into area 4, the ipsilateral olfactory bulb was sectioned serially while the other was subinoculated intracerebrally into a monkey. No lesions were present in the sectioned bulbs and there was no indication of active virus in the subinoculated ones. These findings are in complete agreement with those of Sabin and Olitsky (15) who after intracerebral, intrasciatic, and subcutaneous inoculation found no lesions in the olfactory bulbs.

The second group in Table I concerns the status of nine pairs of olfactory bulbs after intraocular inoculation. Infections of this type run a characteristic clinical course which has been described elsewhere (12), and it has been shown that the virus reaches the brain along the fibers of the autonomic nervous system. The histological picture in the brain indicates an invasion, usually *via* the ciliary ganglion, which is maximal in the midbrain and less intense in the basal portions of the forebrain. Although a slight meningitis was again present around some of the olfactory bulbs of this series, there were no lesions within their substance, indicating that if virus does reach this portal, it produces no reaction in the tissue. Also included in this group are two cases representing the inoculation of other cranial nerves, the vagus and hypoglossus respectively. The technique for neural inoculation has been previously described (16) and consists of moistening the cut end of the nerve with virus emulsion. It has been employed for all inoculations of this type. The animals showed initial bulbar symptoms and later limb paralyses. Their olfactory bulbs were histologically normal.

The third group (seventeen cases) is a composite one containing sciatic, intracutaneous, intraperitoneal, and intraspinal inoculations. They are included under one heading since they all represent infections ascending to the brain from the spinal cord. The Rockefeller MV strain was employed for all inoculations except the intracutaneous and intraperitoneal which were done respectively with the Wallingford virus, 17th generation (17), and an etherized human stool (18). The intraspinal inoculations were performed by direct piqué into the cord. In every instance but two (a non paralytic case showing cord lesions and a case of leg paralysis only), extensive involvement of both arms and legs resulted. A number of cases of the ascending type have already been described (12). They are characterized by a diminution in the severity of the lesions in the rostral portions of the forebrain. Each pair of olfactory bulbs belonging to this group was histologically normal.

The last control group consists of seven animals which received their inoculations intranasally. A wide variety of virus strains was used, ranging from the MV and Wld to four different human stools (19). All the animals were severely paralysed and their olfactory bulbs showed characteristic lesions. Sections of the ciliary ganglia, however, revealed no histological abnormalities. This series thus forms a parallel with those which showed no abnormalities in

TABLE I
Distribution of Lesions after a Single Series of Inoculations

Experiment	Virus	Portal	Outcome	Other portals
6-27	MV	Area 4	Paralysis CNS +	Olfactory bulbs normal
6-39	"	" "	" " +	" " "
6-91	"	" "	" " +	" " "
7-75	"	" "	" " +	" " "
9-39	"	" "	" " +	" " "
9-40	"	" "	" " +	" " "
9-41	"	" "	" " +	" " "
9-42	"	" "	" " +	" " "
9-67	"	" "	" " +	" " "
9-68	"	" "	" " +	" " "
7-87	"	" 5	" " +	" " "
5-92	"	" 6	" " +	" " "
1-09	"	Frontal pole	" " +	" " + ?*
7-26	"	Intraocular	Paralysis Ciliary ganglion +	Olfactory bulbs normal
7-93	"	" "	" " " +	" " "
8-51	"	" "	" " " +	" " "
8-53	"	" "	" " " +	" " "
9-20	"	" "	" " " +	" " "
9-21	"	" "	" " " +	" " "
A8-1	"	" "	" " " +	" " "
A7-5	"	" "	Paralysis	" " "
A7-7	"	" "	"	" " "
A7-4	"	Vagus nerve	Bulbar paralysis	" " "
A1-36	"	Hypoglossal nerve	" "	" " "
A-8	MV	Sciatic nerve	Paralysis CNS +	Olfactory bulbs normal
A2-7	"	" "	" " +	" " "
A6-5	"	" "	" " +	" " "
A6-6	"	" "	" " +	" " "
A8-2	"	" "	" " +	" " "
A8-7	"	" "	" " +	" " "
7-90	Wfd	Intracutaneous	" " +	" " "
8-66	"	" "	" " +	" " "
8-67	"	" "	" " +	" " "
8-68	"	" "	" " +	" " "
8-69	"	" "	" " +	" " "
8-46	Stool	Intrapentoneal	" " +	" " "
8-47	"	" "	" " +	" " "
A9-4	MV	Intraspinal	" " +	" " "
A9-7	"	" "	" " +	" " "
A9-8	"	" "	" " +	" " "
A1-08	"	" "	" " +	" " "

* Bulbs probably infected by direct penetration of inoculum

TABLE I—*Concluded*

Experiment	Virus	Portal	Outcome	Other portals
9-43	MV	Intranasal	Paralysis Olfactory bulbs +	Ciliary ganglia normal
A1-4	Wfd		+	
A1 7†	MV		+	
A3-2	Stool		+	
A4-6			+	
A4-9			+	
A8 3‡			+	"

† Guenon

‡ Chimpanzee

the olfactory bulbs after intraocular inoculation, although the portals are reversed

All of the foregoing cases may be regarded as controls for the observations which follow. They indicate that even in animals suffering paralytic poliomyelitis there are apparent limits to the centrifugal spread of virus in the central nervous system. It is thus possible with reasonable care to produce clinically definite poliomyelitis by a wide variety of routes without the invasion of the first two neurones in the olfactory system. It is also clear that after olfactory inoculation such far flung portions of the nervous system as the ciliary ganglia are not invaded. Whether neurone systems more contiguous to the pathways commonly used by virus in the CNS (12) are involved, depends upon the severity of the initial infection, and other unknown factors.

It now remains to demonstrate to what extent virus invasion may take place in a convalescent monkey through a previously untouched portal. A series of nineteen monkeys has been tested for immunity in this fashion. In the majority of cases the testing inoculation was given by the intranasal route for the obvious reason that it should be the one portal where humoral and neural sources of immunity could be expected to cooperate most effectively. Two possibilities present themselves for the assay of this untouched portal—homologous and heterologous strains of virus. The results of the tests are summarized in Table II.

Heterologous Virus Inoculations—Two Portals

Group A contains three animals, which were reinfected with a heterologous strain of virus. In two of them (9 26, 9 27) the olfactory portal was inoculated with MV virus after previous intracutaneous (abdominal skin) and intracerebral (area 4) inoculations with Wfd virus.

No 9 26 Mar 2, 1939 Abdomen cleaned and an area the size of a half-dollar scraped raw but not bleeding in the gross. 1.5 cc Wfd 8 18—8 19 (17th generation) virus rubbed into this area. Mar 6 Repeated.

TABLE II
Inoculation of Convalescent Animals

First inoculation				Second inoculation				
Experiment	Virus	Portal	Outcome	Virus	Portal	Outcome		
Group A Heterologous virus strains				Different portals				
9 26	Wfd	Intracutaneous	Paralysis	MV	Intranasal	Paralysis*		
9-27	"	Intracerebral	"	"	"	" *		
A4-5	Pest	Intranasal	"	"	"	Fever, ciliary ganglion +		
				Wfd	Intraocular			
Group B Homologous virus strains				Different portals				
A9 3	MV	Below spinal transection	Knee jerk lost L cord +	MV	Intranasal	Paralysis * Olfactory bulbs +		
A9 6	"	" "	Knee jerk lost L cord +	"	"	Paralysis* Olfactory bulbs +		
A9 9	"	" "	Knee jerk lost L cord +	"	"	Olfactory bulbs— Old lesions in medulla		
A1-17	Wfd	Intraocular	Paralysis	Wfd	Intranasal	Olfactory bulbs +		
A1-34	"	"	"	"	"	" " —		
A1-35	"	"	"	"	"	" " +		
A1-74	Pool	Intracerebral	"	Pool	"	" " +		
A1-07	Pool	Intranasal	Paralysis	Pool	Intraocular	Ciliary ganglion —		
A1-12	"	"	"	"	"	" " +		
Group C Identical virus				Parallel portals				
First inoculation				Second inoculation				
Experiment	Virus	Portal	Outcome	Virus	Portal	Fever	Olfactory bulbs	
							Right	Left
A1-26	A8-3	Right nostril	Paralysis				+	—
A1-28	"	" "	"				+	—
A1-31	"	" "	"				+	—
A1-30	"	" "	"				+	+
A1-24	"	" "	"	A8-3	Left nostril	+	+	+
A1-25	"	" "	"	"	" "	0	+	+
A1-27	"	" "	"	"	" "	+	+	+
A1-32	"	" "	"	"	" "	+	+	+

* Paralysis of muscle groups not involved in first attack

- Mar 16 Right leg completely paralysed
- Mar 17 Left leg 5 per cent functional (has a trace of knee jerk—can feebly abduct digits) Right leg as before, arms normal
- Apr 18, 19 Extremities as before—climbs actively with arms only Inoculated intranasally Wfd 9 28 (18th generation) Animal and control remained well.
- May 3, 4 Inoculated intranasally Wfd 9 28 Animal and two controls remained well
- June 3, 5 Inoculated intranasally, fresh Wfd 9-66 (18th generation) Animal and two controls remained well
- June 18, 19 Inoculated intranasally Wfd A1-4 (19th generation) Animal and two controls remained well
- July 12, 13 Inoculated intranasally, fresh MV virus A2 2,¹ 0.5 cc per nostril
- July 16-21 Dromedary temperature spike
- July 22 Right arm zero function, left arm 10 per cent functional
- July 23 Arms zero—left leg as on Mar 17
- July 25 No change—left leg as before Killed
- Olfactory bulbs—each shows extensive recent invasion There were also fresh lesions along with chronic ones throughout the brain
- Summary*—An animal with complete paralysis of the right leg and only a few muscle fibers functional in the left leg following intracutaneous inoculation of Wfd virus, suffered complete arm paralysis after subsequent intranasal inoculation of MV virus There was no further invasion of the lumbar cord The olfactory bulbs showed extensive fresh lesions
- No 9 27 Mar 2, 1939 Inoculated into motor area of cortex cerebri with Wfd virus 8-18—19 (17th generation)
- Mar 8 Temperature 106°
- Mar 12 Extremities in terms of normal strength right arm zero, left arm 60 per cent right leg zero, left leg 60 per cent
- Mar 24 Convalescent Gets around without difficulty Arms and left leg are nearly normal Right leg 5 per cent Inoculated intranasally Wfd 9 28 (18th generation) Remained well
- Apr 18, 19 Inoculated intranasally Wfd 9 28 Animal and two controls remained well
- May 3, 4 Inoculated intranasally Wfd 9 28 Animal and two controls remained well
- June 3, 5 Inoculated intranasally fresh Wfd 9-66 (18th generation) Animal and two controls remained well
- June 18 19 Inoculated intranasally Wfd A1-4 (19th generation) Animal and two controls remained well
- July 12, 13 Inoculated 0.5 cc. per nostril fresh MV A2 2
- July 17 18 Temperature 106.2°
- July 20 Weak and uncertain—barely able to climb

¹ MV virus has been consistently used as a 20 per cent cord emulsion

July 22 Still barely able to climb Right arm 50 per cent, left arm 30 per cent left leg 50 per cent Killed

Olfactory bulbs—recent maximal invasion left, patchy invasion right

Summary—An animal showing extensive paralysis following an intracerebral inoculation of Wfd virus, made a good convalescence except for an almost completely paralysed right leg Following subsequent intranasal inoculation with MV virus it developed fever, and paralysis in the arms and left leg The olfactory bulbs showed extensive recent lesions and there were fresh lesions as well as chronic ones throughout the brain

The remaining animal in Group A was reinfected by the intraocular route following an original olfactory infection

No A4-5 Oct 13, 14, 16, 17, 1939 Received 1 cc per nostril of human stool
Oct 20-23 Temperature 106.8°

Oct 24 Extremities in terms of normal strength Right leg 20 per cent, left leg 10 per cent, arms 100 per cent, still climbing

Nov 30 Gets about actively—biopsy of motor cortex shows characteristic lesions

Feb 8, 9, 10 Arms stronger than previously—legs useless though some movement is possible Inoculated intranasally 1 cc per nostril MV A7-7

Feb 13, 14 Temperature up 2° from level base line—no other reaction Three of four controls paralysed

Feb 20, 21, 23 Inoculated intranasally 0.5 cc per nostril MV A8-2—no reaction Two controls paralysed

Mar 18 0.1 cc. Wfd A6-2 injected into vitreous of left eye

Mar 25 Temperature up 1.5°—no other reaction Control paralysed

Apr 5 Symptomless Killed

Pathological findings Superior cervical ganglia normal Right ciliary ganglion normal *Left ciliary ganglion showed many foci of lymphocytic infiltration and a possibly cuffed vessel* (Fig 1) The olfactory bulbs showed relatively fresh lesions

Summary—An animal convalescent from an intranasal inoculation of human virus, reacted to subsequent intranasal inoculation of MV virus with fever Following intraocular inoculation of Wfd virus there was slight fever but no further paralysis The appropriate ciliary ganglion, however, showed lesions

The foregoing cases show clearly that a highly virulent virus such as the Rockefeller MV strain is capable of almost completely overriding any immunity induced by a relatively weaker strain such as the Wfd if it is introduced by a previously uninvaded portal The single case, A4-5, would indicate that the MV virus probably "took" in the already extensively invaded olfactory bulbs and that the first neurone of the intraocular portal was still susceptible to a third less virulent strain of virus, the Wfd Kessel and Stimpert (7) found that a mild strain inoculated intracerebrally was also capable of producing further paralysis in an animal convalescent from a virulent strain previously given by the intracerebral route

Homologous Virus Inoculations—Two Portals

Group B is concerned with nine tests of immunity by the inoculation of the same virus strain through previously uninvaded portals. This group falls into two categories, that in which two surgically isolated portions of the CNS were inoculated (three animals) and that in which the intact animal was used (six animals).

The first category contains three spinal animals which were inoculated below their transections, subsequently showed signs of poliomyelitis in both legs, and after a 26 to 34 day interval were reinoculated intranasally. Two reacted as though they had never been in contact with virus and became completely paralysed above the transection. The protocols are given below.

No A9-3 Jan 10 1939 Spinal cord transected at lower level of T₆—muscle and fascia inserted between cut ends

Jan 22 Inoculation of 0.4 cc. MV A2-3 in two piqûres into lumbar enlargement

Jan 23 Knee jerks no longer present on either side

Feb 17 Animal in excellent general condition despite several decubitus ulcers over malleoli and pelvic bones. Very active and aggressive despite transection. Inoculated intranasally, 0.5 cc. per nostril with MV virus on this day, and also Feb 18, 19, 21, 23 with MV A7-7 and A8-2

Feb 23 Control paralysed

Feb 27 Control paralysed

Mar 1 Very tremulous—arms still strong

Mar 2 Arms 20 per cent functional—unable to support animal in sitting position. Killed.

Pathological findings. Spinal cord at L₆₋₇ shows loss of practically all cells in grey matter. The process is an old one. The cervical cord shows extensive fresh lesions. Olfactory bulbs show heavy recent invasion on the left, light invasion right.

Summary—An animal in which the spinal cord was transected was inoculated with MV virus intraspinally 12 days later below the transection. Knee jerks were lost but no signs of poliomyelitis developed in the arms. 26 days later the animal was inoculated intranasally with MV virus. It became paralysed above the transection. The lumbar cord showed old lesions, the cervical cord and olfactory bulbs fresh ones.

No A9-6 Jan 11, 1940 Spinal cord transected at T₉. Muscle and fascia inserted between cut ends

Jan 26 Lumbar enlargement inoculated 1.5 cc. MV A2-3 knee jerks and ankle jerks active

Jan 29 Knee jerks + ankle jerks absent

Feb 29 Animal in excellent condition. Active and aggressive—arms very strong—has shown no signs of poliomyelitis above the transection. Inoculated this day and Mar 1, 2 intranasally with 0.75 cc. per nostril MV virus (A8-2 A7-7 8-38, 9-05 pooled)

Mar 5 Temperature has risen from 103° to 106.2°

Mar 7 Control paralysed

Mar 10 Weak and tremulous

Mar 11 Both arms 30 per cent functional, head tremor Killed

Pathological findings Spinal cord at L₆ shows extensive old lesions wiping out practically all grey matter Cervical cord, moderately heavy fresh lesions Right olfactory bulb, heavy recent invasion Left olfactory bulb, perivascular cuffing only

Summary—An animal in which the spinal cord had been transected was inoculated directly with MV virus into the lumbar cord 15 days later Poliomyelitis resulted as evidenced by loss of leg reflexes and lesions in lumbar cord, but the animal remained symptomless above the transection 34 days later intranasal inoculation of MV virus resulted in arm paralysis Fresh lesions were present in the olfactory bulbs and cervical cord

No A9-9 Jan 12, 1940 Spinal cord transected at T₉ Muscle and fascia interposed between cut ends

Jan 25 Removal of right sympathetic ganglia at T₈ 9 10

Jan 29 Removal of left sympathetic ganglia at T₈ 9 10 Inoculation of cord in lumbar enlargement 0.3 cc MV A2-3

Jan 31 Knee jerks and ankle jerks present

Feb 4 Temperature has risen from 102.4° to 105.8° Has low grade infection of incision but is active and alert While being carried for treatment had attack of syncope

Feb 7 Control paralysed

Feb 11 Has been lying down Both arms are weak and the left hand has practically no grasp Virus has entered the cervical cord and probably the medulla² (cf fever and fainting spell of Feb 4)

Feb 23 No change Inoculated intranasally on this day and Mar 1, 2 with MV A8-2, A7-7, 8-38, 9-05—0.075 cc per nostril

Mar 13 No reaction of any sort (Controlled by A9-3 and A9-6) Attempt to remove olfactory bulbs by biopsy abandoned and animal killed

Pathological findings Lumbar cord showed extensive old lesions The medulla and midbrain contained lesions which were lighter in the latter, indicating an ascending infection The olfactory bulbs were badly traumatized so that accurate examination was impossible They contained no heavy lesions but there were some questionable infiltrations

Summary—An animal in which the spinal cord was transected and the sympathetic chains bilaterally interrupted at the same level was inoculated into the cord with MV virus below its transection 15 days later It subsequently developed arm paralysis and showed symptoms referable to a disturbance of the medulla Following intranasal inoculation of MV virus 31 days subsequently it remained symptomless There were very questionable lesions in the olfactory bulbs, but old lesions in the cervical cord and medulla

In the foregoing cases there is no reason to question the first attack of poliomyelitis following cord piqure The lesions present in the lumbar cord

² Progression of virus around a transection of the spinal cord has occurred in eight of eleven animals (44)

could not have been caused by indirect traumatic or vascular injury incidental to the cord transection. The fact that tendon reflexes were present until a few days after inoculation rules out this possibility. Furthermore the obvious chronicity of the lesions with respect to those in the olfactory bulbs and cervical cord indicates that two separate invasions must have taken place. The presence of lesions in the olfactory bulbs removes the possibility that the animals suffered merely an exacerbation of their original disease. Whether the longer incubation periods as compared with the controls indicate a certain degree of immunity cannot be determined from the limited material at hand.

The olfactory portal was deliberately chosen for the testing inoculation because it seemed more physiological than the intracerebral route. The amounts of virus given intranasally were no doubt excessive, but in the case of the second animal (A9 6) no more than that usually employed for a routine inoculation where success must be definitely assured. The relativity of immunity with respect to the dosage which can be tolerated has been brought out by Toomey (4) who reported numerous second attacks in previously paralysed animals following large intracerebral inoculations of homologous virus. Nevertheless when extensive virus dissemination has taken place, the animal may show no paralysis after large doses of the infective agent administered intranasally (2, 20). This point is illustrated also by A9 9 which did not succumb to a second infection and will become clearer in the light of the cases which follow.

Returning to Table II it will be seen that the second category of homologous inoculations employing two different portals of entry deals with two groups of intact animals.

Résumé of Histories of Animals Convalescent Following Intraocular or Intracerebral Inoculations and Reinoculated Intranasally —

No A1 17 Mar 18, 1940 Received 0.1 cc. Wfd A6-2 (15th generation) into vitreous of left eye

Mar 25-27 Fever, followed by partial paralysis of left arm and leg

May 22-24 Extremities in terms of function Arms 100 per cent, right leg 80 per cent, left leg zero Inoculated 0.5 cc. per nostril Wfd A6-2

June 6 No reaction to inoculation Killed Two controls paralysed

Pathological findings Ciliary ganglia show no characteristic lesions (nearly 3 months after intraocular inoculation) The olfactory bulbs show extensive recent lesions (Fig 2)

Summary — See Table II, Group B

No A1-35 Mar 29, 1940 Received 0.1 cc. Wfd virus A6-2 (15th generation) into vitreous of left eye

Apr 7 For several days has had bilateral ptosis, disappearing on excitement and gradually mounting temperature which today reached 105.8°

Apr 13 Prostrate Extremities in terms of function right arm 30 per cent, left arm 10 per cent, legs zero

May 22, 23, 24 Can pull himself into sitting position Right arm 40 per cent, left arm 20 per cent Legs zero except for feeble toe movements Inoculated intranasally 0.5 cc per nostril Wfd A6-2

June 12 Temperature has been more irregular than before the last inoculation, but the arms have gained steadily in strength so that the animal is now able to climb all over the cage No change in legs Killed (Control paralysed)

Pathological findings Right ciliary ganglion normal, left ciliary shows one cuffed vessel and two areas of lymphocytic infiltration Olfactory bulbs show extensive recent lesions of the same character as those of the control (Fig 3)

Summary—See Table II, Group B

No A1-34 Mar 29, 1940 Received 0.1 cc Wfd virus A6-2 into vitreous of left eye

Apr 6 Temperature 105.4° Tremulous—bilateral ptosis

Apr 8 Slight bilateral ptosis All extremities 60 per cent functional

May 22, 23, 24 In excellent condition—runs and climbs without difficulty Extremities in terms of function left arm 100 per cent, right arm 80 per cent, left leg 100 per cent, right leg 70 per cent Inoculated intranasally 0.5 cc per nostril, Wfd A6-2

June 11 Has shown no reaction of any sort Killed (Two controls paralysed)

Pathological findings Right ciliary ganglion normal, left ciliary ganglion shows cuffed vessel Olfactory bulbs normal (Fig 4) Control bulbs show extensive lesions

Summary—See Table II, Group B

No A1-74 Chimpanzee Dec 6, 1940 Inoculated into motor cortex with 0.5 cc cord emulsion from chimpanzee A8-3

Dec 10 Temperature elevated from base line of 101° to 104°

Dec 13 Temperature dropping—weakness of left arm—clumsy and listless

Dec 16 Left arm useless, right arm about 20 per cent functional—barely able to pick up food Legs 40 to 50 per cent functional

Feb 7, 1941 Because of pulmonary tuberculosis animal was anesthetized with nembutal and reinoculated intranasally with the same specimen of virus previously employed, 0.5 cc per nostril, on Feb 7 and 8 Preinoculation physical examination showed considerable recovery Left arm shoulder 15 per cent, biceps 20 per cent, triceps 10 per cent, grasp 10 per cent, tendon reflexes all easily obtained but very feeble Marked muscular atrophy of upper arm Right arm shoulder 25 per cent, biceps 40 per cent, triceps 20 per cent, grasp 25 per cent in fingers only, thumb zero Tendon reflexes all brisk but not forceful Uniform muscular atrophy Legs essentially normal

Feb 14 Despite tuberculosis twice daily temperature records showed a fairly steady base line at 101.5° Temperature began to climb on Feb 10 reaching 102°, and on Feb 14, 103° The next day it dropped to 101°

Feb 17 Right arm reflexes hyperactive—grasp 5 per cent in finger, thumb 0 (Weaker than on Feb 7) Intranasal *rhesus* control showed light paralysis

Feb 20 Left arm biceps 20 per cent, triceps 2 per cent (triceps reflex now very feeble and difficult to get) grasp 10 per cent, flexion at wrist 20 per cent Right arm biceps and triceps 10 to 15 per cent, flexion at wrist 20 per cent, grasp 2 per cent Reflexes all obtained Legs as before Killed

Pathological findings There were fresh lesions in both olfactory bulbs (Fig 5) The cervical cord (C₇ and C₈) showed extensive lesions but it was impossible to be sure that any were of recent origin

Summary—A chimpanzee which was extensively paralysed following an intra cerebral inoculation into the motor cortex was reinoculated intranasally 7 weeks later There was a slight but definite febrile response to the second inoculation and increased weakness in certain portions of the arms Microscopic sections showed fresh lesions in the olfactory bulbs, though a new process could not be established in the cervical cord (Fig 5)

Résumé of Histories of Animals Convalescent Following Intranasal Inoculation and Reinoculated Intracocularly—

No A1-07 Feb 13, 14 15, 1940 Inoculated intranasally as a control for chimpanzee A1-05 Received 1 cc. per nostril per day of a pool of six potent human stools (17)

Feb 18 Temperature 106.4°

Feb 25 Completely prostrate Right leg and tail still 10 per cent functional Neck strong and cranial nerves intact

Mar 25 The animal is incontinent of urine and completely paralysed except for the tail, head neck and diaphragm There are great muscular atrophy and decubitus ulcers over the trochanters which have been arrested by keeping him face down over an inflated automobile inner tube Received 0.1 cc. of A1-05 virus (chimpanzee for which he was control) into vitreous of left eye.

Apr 17 Has shown no reaction attributable to second inoculation Killed (Control paralysed lesions in left ciliary ganglion)

Pathological findings Right ciliary ganglion normal left ciliary ganglion shows two rather large cuffed vessels at its margin and focal accumulations of round cells within it (Fig 6) Olfactory bulbs show definite old lesions but much of the debris has been cleared up

Summary—See Table II, Group B

No A1-12 Feb 26-29, Mar 1 1940 Inoculated intranasally (1 cc. per nostril) stool of chimpanzee A1-05

Mar 4 Temperature 105.6°

Mar 9 Prostrate—no function in arms and legs

Mar 25 Completely paralysed except for head, neck, diaphragm, and tail—marked muscular atrophy and contracture Received 0.1 cc. chimpanzee virus A1-05 into vitreous of left eye

Mar 27 Temperature up from base line of 102° to 104° Eye red lid swollen

Mar 28 Eye more red—lids swollen, exudate Temperature normal

Mar 29 Eye much better, still opaque but lids nearly normal

Apr 8 Found dead Had shown no clinical signs of poliomyelitis although control came down 1 week previously

Pathological findings Ciliary ganglia normal Bulbs show old lesions
Summary—See Table II, Group B

The foregoing series of cases shows a second invasion of homologous virus into a new portal in four out of the six. In only one instance was this "second attack" accompanied by any clinical manifestation. There is a suggestion that some of the cases of second paralytic attacks following intracerebral inoculation reported by Toomey (3, 4) fall into a category intermediate between our spinal animals and these which had no further paralysis. Some of his animals which were convalescent from subserosal and intrasciatic inoculations showed little resistance to paralysis from relatively small intracerebral inoculations. This state may have been favored by a light dissemination of virus from the spinal cord rostrally into the brain. In the present instance it seems fairly clear that the virus was not effective at any point beyond the convergence of the pathways from the new portal with those in the brain which had already been invaded. The difficulty of establishing the existence of fresh lesions in the brain discouraged attempts in this direction, but it is not impossible that the virus made further advances without producing symptoms. It would be desirable to repeat the experiments with a series of lightly paralysed convalescent animals in an effort to quantitate the amount of invasion necessary to prevent further paralysis from a given testing dose.

A suggestive case of this sort is found in a chimpanzee A4-8, which received by stomach tube 25 cc. of supernatant fluid from a pool of six potent human stools. On the 6th day following inoculation his temperature rose from a base line of 101–102° to 104.4°. No other abnormalities were noted but a lumbar puncture was not done. The animal subsequently was given 70 cc. of the same pool by stomach tube without further reaction. 8 months later he contracted a partial left facial paralysis after receiving 2 cc. of the same stool pool by mouth on 3 successive days. The lesions were sharply localized to the medulla, centering about the nuclei of the LT fifth and seventh nerves (42).

Homologous Virus Inoculations—Parallel Portals

Table II, Group C, indicates the findings in a small series of animals which are regarded as suggestive that inoculation of one olfactory bulb leaves the other susceptible to subsequent invasion. We shall give a résumé of the histories and advance an interpretation for what it is worth—possibly not a great deal in view of the nature of the problem and the few cases available.

Eight animals were inoculated into the right nostril with a single instillation of 0.5 cc. of a very virulent chimpanzee stool. All subsequently became paralysed (two were prostrate, one almost helpless, and the rest ambulatory). The two prostrate animals and one other were killed immediately as controls. 6 weeks later the nearly helpless animal had to be killed. The olfactory bulbs

of these four animals which served as controls were examined in serial sections. Three showed unilateral lesions. 3 months later the four remaining animals were inoculated as before, but into the left nostril with 0.5 cc. of stool from the same specimen previously employed. Within a week three of the animals showed fever between 104.8° and 106° but there was no extension of paralysis over a 2 week observation period, although one control became prostrate. Examination of the olfactory bulbs revealed bilateral lesions in every case,—a ratio differing markedly from that of the controls. It was thus strongly suggested that in the twice inoculated animals two separate invasions had taken place, the first through the right olfactory bulb, resulting in paralysis, and the second *via* the left, producing fever and lesions on this side. Perhaps the most confusing aspect of the whole problem was connected with the age of the lesions in the bulbs. There were available several pairs of olfactory bulbs from 2 to 3 month convalescent animals inoculated with various strains of virus and one bulb of a 6 week convalescent animal from the series in question. These indicated that inflammation is resolved relatively rapidly in the olfactory bulb, so that in 6 to 8 weeks little is left beyond remnants of cuffing in the marginal fiber layer and diffuse gliosis of the molecular and glomerular layers. Rod cells are no longer abundant at this stage and the mitral cell losses merely appear as vacancies in their respective layer. In the olfactory bulbs of the reinoculated convalescent animals it was not surprising to find massive cuffing with fresh neuronophagia and cellular infiltration on the side inoculated last, but to find these changes bilateral in two cases was totally unexpected. Since the series did not provide control material of the exact age of the bulbs in question, one can do little more than hint that fresh invasion had taken place on the side of first inoculation as well as on that inoculated more recently. It is hoped that further study will clarify this very important point.

Homologous Virus Inoculations—One Portal

It is generally true that in the monkey a paralytic attack of poliomyelitis following intracerebral or intranasal inoculation protects against paralysis from any but massive inoculations of the same virus by the same portal (3, 4, 7-11). Nevertheless, there are several nuances of this apparent fact which are worthy of consideration. For example one intracerebral inoculation is taken as the equivalent of another, but this is not necessarily true, since virus does not distribute itself uniformly through the entire cortex. This factor is probably of no practical importance as regards the immunizing and testing inoculations usually employed in the laboratory, since the overwhelming quantities of virus used ensure wide dissemination throughout the brain. However, it is conceivable that carefully controlled experiments closer to the monkey's threshold of susceptibility might reveal that two infections could

take place from cortical areas possessing different projection pathways, or even through a single olfactory bulb. This becomes a more realistic possibility when one considers human infections which may well occur from small quantities of virus entering the CNS on different occasions.

Heterologous Inoculations—One Portal

Several investigators attest the lack of immunity which intracerebral or intranasal inoculation of one strain may confer to another inoculation of a different strain (2, 4-9, 23).

A good illustration of this phenomenon is seen in monkeys A1-76 and A1-73. These animals both received intranasal inoculations of the same pool of human stools. They were both paralysed to about the same degree but remained ambulatory despite involvement of all extremities. 2 months after paralysis was first noted A1-76 received three daily intranasal inoculations of MV virus (0.5 cc per nostril). The temperature went up to 107° on the 4th day and remained elevated in a dromedary spike for 1 week. Toward the end of this period the animal became so weak that it was barely able to climb. Examination of the olfactory bulbs showed massive invasion on both sides (Fig. 7). The olfactory bulbs of A1-79 which was allowed to live for the same period showed almost no lesions though many mitral cells had disappeared. Fig. 8 was taken from an area in one bulb which represented the maximal changes. It does not even approximate in severity the lesions found in the animal suffering the second attack.

Some virus strains appear to have a marked ability to override previous immunity, but according to Kessel and Stimpert (7), this is not a property of their virulence. It is again not unlikely that many animals which do not show additional paralysis may nevertheless suffer extension of lesions. A case in point is monkey A4-5 which has already been listed in Table II, Group A. The animal was given an intranasal inoculation of human stool which resulted in almost complete leg paralysis. 4 months later it received three daily intranasal inoculations of MV virus. On the 6th day following the first of the second series of inoculations its temperature rose from a steady base line of 102° to 105° and remained between 105° and 106° for 3 days. There was no visible extension of paralysis, and the animal was subsequently given an intraocular inoculation of Wfd virus to which it reacted with a slight temperature elevation. It was killed 2 months after the intranasal inoculation of MV virus, and 6 months after its original intranasal inoculation. The olfactory bulbs showed relatively fresh lesions which could scarcely have been present for 6 months. Flexner (2), and Lennette and Gordon (8) have recorded similar cases of animals which have suffered two paralytic attacks after two successive series of heterologous intranasal inoculations.

It now remains to consider the possible immunizing effects of virus which is

brought into direct contact with peripheral nerve axones but which does not produce a temperature reaction or any other sign of disease in the animal. This situation was encountered in six monkeys which remained symptomless following the moistening of the cut end of one sciatic nerve in a virus emulsion of demonstrated potency. Infection did not result because the neurones involved had been rendered temporarily refractory by repeated previous section of the nerve (41). The present point concerns the fact that probably every axone in the sciatic nerve was brought into protoplasmic contact with active virus, and that the transient state of resistance in the nerve thus permitted a much more extensive contact between virus and nerve tissue than would have been possible in the intact animal. The whole procedure might be considered as a sort of supervaccination of the nervous tissue together with a considerable amount of contact between the virus and the subcutaneous tissues. Nevertheless there was no evidence of virus proliferation.

Several weeks after this type of "vaccination" the animals were found readily susceptible to infection. Two became paralysed after the drastic procedure of exposing the opposite sciatic nerve to virus, but the remaining four were severely paralysed following routine intranasal inoculations. Furthermore there was no indication of increased resistance in the cells which had been in contact with virus. The series suggests that no general immunity results without overt virus activity.

DISCUSSION

The experiments just reported are not concerned primarily with humoral antiviral factors. Neither laboratory workers nor clinicians can agree on the rôle of serum antibodies in immunity to poliomyelitis (20-33). At best the degree of protection afforded by various methods of vaccination against intracerebral or intranasal inoculation is slight. Many of the observed inconsistencies may result from the fact that humoral protection and antibody titer vary not only from one individual to another but also in different tissues. The tests for immunity usually employed in the *rhesus* monkey have not brought out these subtle but important differences. It has been assumed throughout our own experiments that in each instance a more than adequate antigenic stimulus must have been provided by the first attack of poliomyelitis and that circulating antibodies were present. Nevertheless despite the fact that the intranasal portal was used for the testing of immunity, it is possible that no opportunity was provided for the participation of humoral factors, since this type of inoculation brings virus into very intimate contact with nervous tissue in which it is rapidly fixed and thus removed from the action of antibody (43). Such a situation has been unavoidable because of the limitations imposed by the use of the *rhesus* monkey which can be infected readily only by direct neural or intranasal inoculation. In all probability these experiments have

been dealing primarily with a type of local immunity which appears to be largely a function of actual contact between virus and susceptible nerve cells (34). They have defined more clearly than heretofore the character of this immunity, showing it to be determined by the movements of virus along nerve fiber pathways. To what extent it may be modified by humoral factors is not at the moment apparent. Very delicately graded tests of the immunity of the central nervous system will be required to demonstrate such a relationship.

It is by no means clear how much of the experimental work on *rhesus* monkeys can be applied to the problem of immunity to poliomyelitis in man. There is little doubt that most individuals do acquire an effective immunity with increasing age and epidemiological evidence favors the idea that this is produced by contact with active virus, rather than by mere physiological maturation or other metabolic factors. The concept of "abortive" poliomyelitis as an immunizing agent, however, is based chiefly upon the postulate of a general immunity of the CNS produced by a limited invasion of virus from a single portal of entry. Actually nothing is known concerning the amount of invasion which may take place in human abortive and non-paralytic forms of poliomyelitis or their antigenic effects. Our own findings suggest that as regards the central nervous system an immunity of this type might not be very substantial since its influence probably would be restricted to the region of immediate exposure. Recent studies of human pathological material point increasingly to the utilization of several portals of entry in man (35-40), so that in order to produce effective immunity an antecedent attack must conceivably close a variety of portals leading to the neuraxis along various peripheral nerves. At the moment one can do no more than speculate on the means by which this might be accomplished.

There is no reason to believe that the central nervous systems of man and the *rhesus* monkey differ fundamentally in their reaction to poliomyelitis virus. The outstanding disparities between these two forms relate to the portals of entry. Thus the monkey, in which practically the entire nervous system is susceptible to virus, presents a very restricted portal of entry, the olfactory area. In this animal, however, it has been shown that paralytic poliomyelitis does not necessarily close this portal "from within" and that a second attack, whether paralytic or not, seems to depend upon the strains of virus used and the degree to which virus was disseminated through the neuraxis during the first exposure. The closure by an abortive attack of *multiple* portals of entry from within would therefore present even greater complications, involving extensive dissemination of virus in the neuraxis without the production of paralysis. It thus seems more logical to conclude that such immunity as can be demonstrated in the *rhesus* monkey by intracerebral in-

oculation has little bearing upon the problem of human immunity and that the immunity which is presumably produced by subclinical virus activity in man comes by the closure of the portals from without, either through the participation of humoral factors, or not inconceivably by alterations in the mucous membranes or other barriers against the entrance of virus into the nervous system

SUMMARY

1 It was found in forty *rhesus* monkeys that intracerebral, intraocular, intracutaneous, intraperitoneal, intraspinal, and neural inoculations of polio myelitis virus produced no lesions in the olfactory bulbs despite the fact that the animals contracted pronounced paralyses. This indicated that the virus could be restricted to certain neuronal systems.

2 Similarly intranasal inoculation of seven animals produced no lesions in the ciliary ganglia.

3 Two monkeys convalescent from an intracutaneous and an intracerebral inoculation respectively had further paralyses after intranasal inoculation of heterologous virus. A third animal convalescent from an intranasal inoculation showed extension of lesions after intranasal and intraocular inoculation with heterologous virus.

4 Two spinal animals in which an attack of poliomyelitis was limited to an isolated segment of spinal cord, contracted typical paralyses in the previously uninvaded portions of the CNS following intranasal inoculation of homologous virus.

5 Four of six convalescent monkeys showed extension of lesions but no clinical signs after homologous virus inoculation through a previously uninvaded portal.

6 Four animals convalescent from a unilateral intranasal inoculation showed evidence of new invasion in the opposite olfactory bulb but no extension of paralyses following a second inoculation of homologous virus into the appropriate nostril.

7 Two animals had second attacks after heterologous second inoculations. The intranasal portal was employed for both exposures.

8 It thus seems apparent that in the *rhesus* monkey a second attack of poliomyelitis, whether paralytic or not, seems to depend upon the strains of virus used and the degree to which virus is disseminated through the neuraxis during the first exposure.

9 The above experimental data emphasize the difficulty of utilizing the *rhesus* monkey for experiments seeking to elucidate the mechanisms of immunity in man and suggest that human immunity to poliomyelitis does not result from immunization of the nervous system but rather is the result of some

process which prevents infective quantities of active virus from reaching nervous tissue

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EXPLANATION OF PLATES

PLATE 7

FIG 1 The left ciliary ganglion of A4-5, an intranasal convalescent monkey which was subsequently inoculated into the vitreous of the left eye with heterologous virus. Note lymphocytic foci which are the characteristic of virus invasion in this tissue.

FIG 2 Olfactory bulb of A1-17, a convalescent from an intraocular inoculation subsequently given homologous virus intranasally. Compare with Fig 4 which shows a normal olfactory bulb. Note dense cellular infiltration and complete loss of mitral cells. $\times 60$

FIG 3 Olfactory bulb of A1-35, a convalescent from an intraocular inoculation subsequently given homologous virus intranasally. Note heavy perivascular cuffing and areas of neuronophagia in mitral cell layer. $\times 60$

FIG 4 Olfactory bulb of A1-34. Treated as in the above cases A1-17 and A1-35 but showing no lesions. Note normal appearance of mitral cells in their respective layer and the acellular character of the molecular layer. $\times 60$

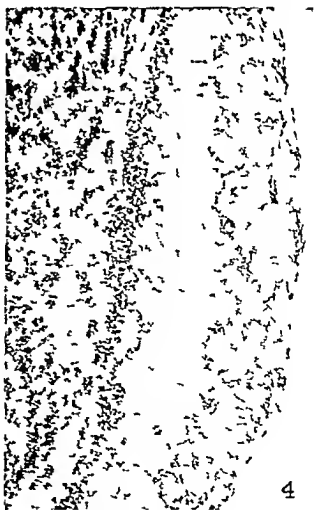


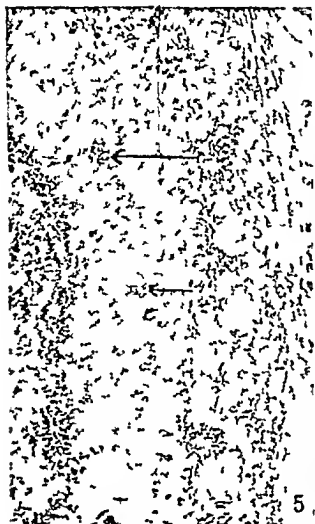
PLATE 8

FIG 5 Olfactory bulb of chimpanzee A1-74 The animal was convalescent from an intracerebral inoculation and subsequently received homologous virus intranasally Note clumps of phagocytes (designated by arrows) surrounding scattered mitral cells in the molecular layer $\times 60$

FIG 6 Left ciliary ganglion of A1-07, a convalescent from an intranasal inoculation subsequently receiving homologous virus in the vitreous of the left eye Note focus characteristic of invasion in this tissue $\times 60$

FIG 7 Olfactory bulb of A1-76, a convalescent from an intranasal inoculation subsequently receiving heterologous virus by the same portal Note the massive character of the invasion Compare with control (Fig 4) $\times 60$

FIG 8 Olfactory bulb of A1-79, a convalescent from an intranasal inoculation It received the same virus as A1-76, suffered approximately the same amount of paralysis, and lived the same number of days, but received no second inoculation The cuffed vessel represents the maximal amount of change which could be found in the olfactory bulbs after this period of convalescence $\times 60$



THE CAPACITY OF THE RENAL VASCULAR BED IN HYPERTENSION

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During the past 80 years there have been many reports of perfusion studies of human kidneys (1, for literature, 2, 3). As a rule the kidneys were perfused as soon as possible after death, with water, serum, saline, or acacia solution. The observed rates of flow, at 100 mm Hg, were from 1 to 2.5 cc per gm per minute in normal kidneys. Correcting for differences in viscosity of the solutions used, these studies suggest a possible blood flow of less than 150 cc (3) to as much as 400 cc (2) per minute for the kidneys of an adult. While this seems high in relation to blood flow in the arm during intense hyperemia (about 0.6 cc per gm per minute) it is less than one third the blood flow through the kidneys during life as estimated by diodrast clearance (4).

The growing interest in renal vascular disease as a possible cause of arterial hypertension makes it highly desirable for pathologists to have available direct measurements of the renal vascular bed as well as morphologic studies of the large and minute vessels. A satisfactory method must yield values comparable with those obtained in similar groups of cases by diodrast clearance. In postmortem material it is obviously impossible by perfusion to duplicate the flow during life, but by proper preparation of the specimens the maximum carrying capacity of the vessels can be measured.

Methods

To measure the maximal rate of flow it is necessary to allow rigor of the blood vessels to pass off, or to break it up by perfusion at high pressure, but such procedures hasten the rate of edema formation in the tissues and the vessel walls. To avoid the decline in flow consequent on edema formation we have used an oil rather than a watery solution. With this technique rates of flow as high as 10 cc per gm per minute, at 100 mm Hg were obtained in young adults although the fluid used, kerosene, has twice the viscosity of saline. Correcting for relative viscosity this method yields figures for possible blood flow well above those obtained by diodrast clearance in people of the same age group.

In most instances but one kidney was used, but only if the other was comparable in weight and structure. In 15 hypertensive cases both kidneys were perfused. The kidney, perirenal fat, renal vessels, and half of the aorta were removed *en bloc*, and the renal artery or arteries cannulated through the aortic ostia. The largest possible snug fitting thin walled metal cannulae were used, these ranged from 2 to 4.7 mm inside diameter. Occasional accessory vessels were cannulated with 1 mm cannulae. Heavy clamps were applied on the perirenal fat, and small leaks were caught with hemostats while perfusing with kerosene at 200 mm Hg. A pneumatic pressure system was used which maintained constant pressure during perfusion, as shown by a mercury manometer. Neoprene tubing was not altered after months of exposure to the oil. The flow from the system with no organ in the circuit was 5100 cc per minute at 100 mm Hg with the largest, 1500 cc per minute with the smallest cannula.

TABLE I

	Flow of kerosene at 100 mm Hg pressure	Kidney weight total for body	Mean blood pressure (S + D)/2	Possible renal blood flow	'Cor- rected' blood flow
	cc/gm/min	gm	mm Hg	cc/min	cc/min
Normal individuals, 18-32 yrs	8.3	370	97	2080	2960
Normal individuals, 46-60 yrs	5.7	390	99	1580	1950
Normal individuals, 61-68 yrs	5.7	310	101	1250	1500
Hypertensives, 38-60 yrs	5.2	350	158	2010	2440
Hypertensives, 61-84 yrs	4.3	304	150	1370	1560

Average values for perfusibility of kidneys of non uremic patients. The methods for calculating "possible renal blood flow" and "corrected blood flow" are given in the text. The former represents the flow of whole blood at the patient's mean blood pressure, calculated from perfusion rate with kerosene at 100 mm Hg. The "corrected flow" is obtained similarly after the observed rate of flow is corrected by subtracting the resistance of the perfusion apparatus from the total resistance of kidney and apparatus. It is apparent that the perfusibility of normal kidneys is only slightly greater than indicated by the data reported by those who have used diodrast clearance, and that there is a distinct fall with age and with arterial hypertension.

Before timing the rate of flow the kidneys were kept for 24 hours after death at 4°C, and at 37°C for 4 hours, in order to allow rigor to pass off, and they were perfused with one liter of kerosene at 200 mm Hg to break up the remaining rigor and wash out the vessels thoroughly. Actually, blood remains in some glomerular loops and a few intertubular capillaries even after perfusing with several liters of kerosene. Since kerosene and blood do not mix, it seems probable that where a large plexus of vessels opens out of a single narrow inlet, as in the glomerulus, the kerosene pours through some channels and traps blood in a few. It was found that perfusion repeated at 48 hours gave no higher rate than at 24, but the lower time limit for maximal perfusion rates was not explored. The rate of flow was timed from a calibrated bottle, with a stop-watch, at 160 mm, then at 100, again at 160 and 100. Occasionally a third pair of observations was made if the second did not check with the first, which in such cases was always slower. Usually the duplicates checked within 5 per cent.

On the average the resistance (Pressure/Flow) was 6 per cent higher at 100 mm than at 160. Individual variation was from 0 to 16 per cent, but the average in normal and hypertensive individuals and at different ages did not differ significantly.

It should be noted that the perfusion system itself was not free from resistance. This introduces little error in cases where flow is less than one liter per minute, but in the rapid flow of young normal individuals the error rises to as much as 50 per cent. Thus the fastest flow 10.5 cc. per gm. per minute, becomes 16 when recalculated subtracting the resistance of the system. We have followed the usual custom of not correcting for this error which tends to minimize the actual spread between the fastest and slowest rates. In Table I, however, corrected average figures are also given.

After washing out the kerosene with warm salt solution, lead carbonate in warm 6 per cent gelatin was injected, the kidney chilled with pressure at about 140 mm Hg in the injection system, and roentgenograms were made with the kidney and vessels intact and again after splitting the kidney lengthwise. This gave a reliable check on the completeness of injection, the state of large branches, and the amount of tissue to subtract if a small accessory artery was missed. Judging by differences between injected right kidneys and non-injected left kidneys, they increased 10 to 15 per cent in weight during perfusion and injection. In this laboratory the renal pelvis and pelvic fat are not trimmed out before weighing.

Material Studied—While recognizing the desirability of using kidneys from coroners' cases in which death occurred quickly from violence or poison, as the normal controls, we have used the best available material—kidneys from patients dying of disease in which the kidneys were not directly involved and only from those with normal heart weights, and several blood pressure records before premortal circulatory failure. None had systolic levels over 140 or diastolic levels over 85. In the hypertensive group all had cardiac hypertrophy, systolic levels over 170 and diastolic over 90 on all observations prior to final circulatory failure, which in many was associated with cerebral or coronary arterial accidents. None was considered uremic unless the blood urea was more than 100 mg. per cent in the last week of life. There were relatively few women among the normal controls; the flow per gram of kidney apparently was in the same range as in males of the same age.

RESULTS

Effect of Age—The kidneys of older people, including those with low normal arterial pressure, show variable degrees of disseminated focal atrophy of the renal cortex, associated with arterial disease, chiefly atherosclerosis and reduplication of the inner elastic lamellae. Such changes are more striking in hypertensives, yet in some of these (markedly hypertensive in a few cases for many years) scarcely any gross change and only rare hyalinized glomeruli are seen. However even in normal individuals with minimal histologic renal changes and those who are senescent rather than senile, the capacity of the vascular bed of the kidney decreases strikingly with age (Table I and Fig. 1).

It seemed desirable to interpret our results in terms of blood flow in order to compare them with reports on diodrast clearance. To correct for the difference in viscosity of blood (4 at 37°C) and kerosene (2 at 20°C), we per

fused one kidney with kerosene and then with a mixture of cosmetic oil and kerosene (viscosity 4). The decrease in flow through the tortuous renal vascular bed on doubling viscosity is not 50 per cent, as would be the case in a straight tube, but only 30 per cent. Using this factor, the observed kidney weight, and mean blood pressure, $S + D/2$, of each patient, we calculated a

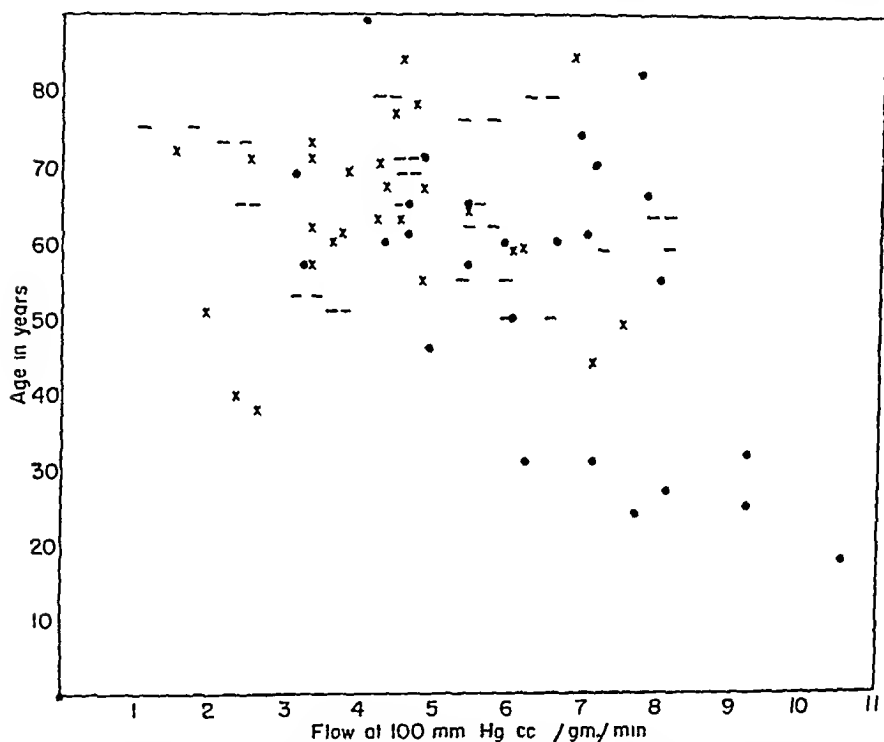


FIG 1 Kerosene flow through kidneys postmortem ● = normal kidney from patient without hypertension, X = kidney from hypertensive patient without uremia, -- = two kidneys from hypertensive patient without uremia

“possible renal blood flow” These figures are not corrected for surface area, but the average for a group may be compared with figures obtained on living men with diodrast (4). We also give a further “corrected” figure, where the resistance of the perfusion system is taken into account, rather than being ignored as is the usual custom. This is given to show the absolute maximum which can be computed if all factors are considered, and also for comparison with figures obtained in the conventional way which assumes that the pressure at the entrance to the main artery is that at the surface of the fluid in the perfusion bottle.

The reported diodrast clearances (4) on fourteen men 18 to 32 years old indicate a blood flow in life of 1390 cc, our seven kidneys in this age group yield a figure for "possible blood flow" of 2080 cc. For thirteen men, aged 45 to 60, the diodrast renal blood flow figure was 1018, 26 per cent below that of the younger group, while kerosene perfusion indicates a "possible flow," in the group 45 to 60 years old, of 1580 cc, 24 per cent lower than in the young adults

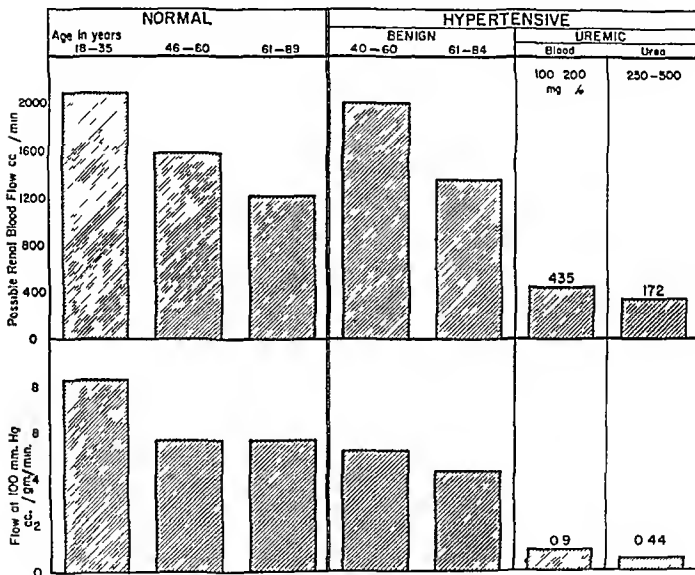


FIG 2 Average values for kerosene flow postmortem, and "possible renal blood flow" calculated as described in Table I. Renal perfusibility decreases with age, is somewhat lower in hypertensives, is greatly diminished in most uremics.

The fact that similar changes with age are apparent in both groups, and that the kerosene method gives a figure 50 per cent higher than the diodrast flow as contrasted with a figure 60 to 80 per cent lower in the reported aqueous perfusions gives us some confidence in the validity and value of the method we have used. The decrease in total flow, in normal kidneys, is 40 per cent between young adults and those past 60 years. In those 60 to 89 the further

decline over the rate of flow in those of 45 to 60 is associated with the decrease in renal tissue, since the flow per gram of kidney at 100 mm Hg remains constant in the two older groups

Hypertension with Normal Blood Urea—The “possible renal blood flow” appears to be greater in these hypertensives as compared with normal individuals of the same age groups, but it is probable that the true mean pressure, in hypertensives, is not so high as that calculated by averaging systolic and diastolic levels. The curves showing percentile distribution of various levels of renal vascular resistance in normal and hypertensive individuals show that about 75 per cent of such hypertensives have resistance in the normal range (Fig 3). Indeed the average kerosene flow per gram, at 100 mm

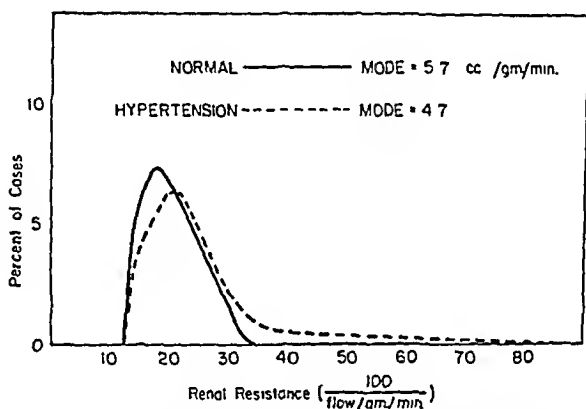


FIG 3 Distribution of values for resistance in renal vascular bed in normal and in hypertensive patients without uremia. While most hypertensives fall in the normal range, a few have slight to marked increase in resistance to flow.

Hg, is only 11 per cent less in the hypertensive groups, although an occasional case shows a level one-half the lower limit of the normal.

Marked Occlusion of Renal Artery and Its Main Branches—Moderate narrowing at the ostia of the renal arteries is often encountered in patients with atherosclerotic plaques in the abdominal aorta. In some of the renal arteries from normal or hypertensive patients such plaques were present, and in two the narrowing was so marked that only a 2 mm cannula, instead of the usual 4.0 or 4.7 mm cannula, could be used. The rates of flow were not low, one of them had the same flow per gram as its mate with a fully patent artery. This merely confirms the observation (5) that narrowing of a short segment of an artery must be very marked before the blood flow is reduced. In addition to this we have observed, in the past 1400 autopsies, five cases with very marked narrowing of the main renal artery or occlusion of one of its large branches. All had a marked atrophy of the kidney or part of the kidney affected but in

only one did the findings seem comparable with Goldblatt hypertension in the dog, in the other four fairly recent thrombosis or hemorrhage in a plaque was present, renal sclerosis distal to this was similar to that elsewhere, and there was a history of years of hypertension. We interpret these as cases in which renal arterial accident supervened in hypertension just as a coronary or cerebral arterial occlusion might have occurred. Such accidents to renal arteries may lead to a new and more severe hypertensive mechanism, and in one case this seemed to have been the case. In three cases only one third to one-half of a kidney was affected, and there was no evidence of a recent rise in pressure.

Uremia—In one case of amyloid disease, with a rapid terminal rise in blood urea to 300 mg per cent, the "possible renal blood flow" was calculated at 1400 cc per minute, the flow of kerosene per gm, at 100 mm was 2.5 cc per gm per minute. In a case of myeloma kidney, with hypertension, the "possible renal flow" was 660 cc, and in a case of polycystic kidneys (total renal weight 4.6 kilos) the possible flow was 620 cc. In the other three patients with blood urea over 250 mg per cent, there was chronic glomerulonephritis and total "possible flow" averaged 172 cc. There were seven cases with blood urea between 100 and 250 mg per cent. One had periarteritis, two chronic pyelonephritis, and four severe renal arteriosclerosis, with scarred and shrunken kidneys. The possible flow in these cases averaged 435 cc per minute. The fastest flow in this group is about the same as the slowest flow in the hypertensives, but uremics with chronic renal disease obviously have much lower possible rates of blood flow, on the average, than even elderly hypertensives (Fig. 2).

DISCUSSION

Aside from Kimmelstiel's (2) mention of the fact that normal kidneys from patients under 40 years old all gave flows of 250 to 400 cc saline per minute, while older ones ranged from 110 to 400 cc, the literature on renal perfusion does not mention an age correlation with vascularity of the kidney. Moore (8) has recorded a decline in total glomeruli per kidney of 8.5 per cent in persons of the 45 to 60 year group as contrasted with the 18 to 35 year group. But in perfusion of the kidney with kerosene it is apparent that the vascular bed decreases about one-fourth between maturity and late middle age in the average person. The same decrease also is seen in the renal blood flows of normal men, calculated from diodrast clearance (4) and in the heart, perfused with kerosene (9). Some individuals do not show any reduction in vascular bed even at 70 or 80, they are like the men who pass three score and ten with no bald spots and few grey hairs. After 60 there seems to be little decrease in blood flow per gram of kidney in normal people, although decrease in kidney weight becomes quite striking in senility.

As in previous studies in which the large renal arteries were studied by roent

genograms and histologic study after an injection mass had set at arterial pressure, significant atherosclerotic narrowing of large renal arteries was uncommon in this series of cases. In a fairly large proportion significant narrowing probably occurs late in hypertension as a result of atherosclerosis and hemorrhage in plaques, the latter accident perhaps being related to the hypertension. Observations based on renal arteries constricted by rigor or fixation have led to wholly erroneous estimates of the frequency of significant narrowing (6). In vessels not fixed while distended at arterial pressure even a small plaque seems to obstruct the lumen.

So far as the relation of renal arteriosclerosis and reduced vascular bed to arterial hypertension is concerned, our results agree essentially with the classical studies of Kimmelstiel (2), whose careful histological correlations we have not repeated. Uremic patients with hypertension have very much slower rates of flow, on perfusion after the kidney has passed out of rigor, than do non-uremic hypertensives. In Kimmelstiel's work the average normal flow, at 140 mm Hg, was 231 cc per minute, the average in hypertensives ranged from 199 in the group with no arteriolar sclerosis to 175 in those with marked arteriolar sclerosis but none of the pathological stigmata of "malignant sclerosis." In those with such stigmata, the flow, as in those with the clinical features of "malignant sclerosis," averaged just over 50 cc, those with chronic glomerulonephritis just below 50 cc. While our absolute values are very different, the trend is similar, and contrary to the findings of Christian, Schlesinger, and Myers (3), who apparently perfused kidneys immediately post-mortem, probably before rigor had passed off, and found little difference between uremic and non-uremic hypertensives.

On the whole the results of renal perfusion indicate that hypertension frequently occurs without any significant reduction in the vascular bed, and not infrequently with a vascular bed potentially as capacious as that of a young adult. The average resistance to flow through the renal tissue of such hypertensives is only 20 per cent greater than in normal individuals, but this is due to the fact that old hypertensives have more renal vascular disease than comparable controls (producing in our material a perfusibility 32 per cent lower in the hypertensives over 60), and even at early ages a few hypertensives have kidneys with marked vascular change and reduced capacity for flow. We interpret the evidence, in the light of other observations and particularly the phenomena encountered in experimental renal hypertension, as indicating that hypertension may accelerate and initiate degenerative changes in the retinal and renal arterioles, and to lesser degree changes in larger vessels and other organs. Therefore much more severe vascular changes and greater reduction in vascular bed will be observed in some hypertensives than in any normal individuals. Of particular interest were two pedigreed hypertensives, always showing levels of 200/120 or higher over more than 10 years, who had prac-

tically normal kidneys and renal vessels, and capacity for flow in the upper normal range. In other words hypertension is usually not associated with renal arterial disease at its onset, and is usually not accompanied by significant reduction in renal vascular bed. It can, and often does cause renal arteriosclerosis to progress to the point where the vascular bed is narrow and uremia begins, but it may exist for years without affecting these vessels. While the kidneys may play a part in all cases of hypertension, it is only in a small group that renal disease, usually inflammatory, is the primary process.

SUMMARY

By using kerosene and avoiding postmortem rigor one can obtain perfusion rates in kidneys nearly five times faster than those reported by observers who perfused kidneys immediately post mortem with saline solution, only half as viscous as kerosene.

The results obtained by kerosene perfusion indicate possible renal blood flow 50 to 100 per cent greater than that measured by Smith and his coworkers (7) in living men by diodrast clearance under normal conditions, and about as high as those observed in febrile subjects. Like the diodrast method, kerosene perfusion shows a striking decrease in renal vascular bed between early maturity (age 18 to 35) and senescence (45 to 60). This decrease is about 25 per cent.

Most kidneys from patients with hypertension without uremia have vascular beds in the normal range, but a few show great decreases in capacity for blood flow. This evidence is interpreted as another indication that renal arteriosclerosis is often a result, rarely a cause of hypertension. Significant occlusion of large renal arteries is rare.

Uremia due to amyloid may occur with no significant decrease in renal vascular bed, but the uremia of renal sclerosis, glomerulo- or pyelonephritis is associated with reduction of vascular bed to very low levels.

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THE CAPACITY OF THE CORONARY BED IN CARDIAC HYPERTROPHY

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Until diodrast clearance was used to study renal blood flow, there was no yardstick of normal blood flow through human organs during life to compare with the rates of flow observed during perfusion postmortem. It now is evident that the studies of renal perfusibility carried out soon after death with aqueous solutions yielded values, at 100 mm Hg only 10 to 40 per cent as great as the flow calculated for young adult male kidneys excreting diodrast. On the other hand, waiting until rigor passes off, perfusing at high pressure to break up rigor, and using kerosene as a perfusion fluid yields values, at 100 mm Hg, well above diodrast clearances (1). It therefore seemed worth while to use the same technique on the coronary arteries in hope of obtaining information on the vascular bed of hypertrophied hearts to supplement that obtained by morphological studies (2).

Methods

The technique was essentially the same described for kidneys (1). The cannulae thin walled metal, and of the largest, snug fitting caliber were introduced through the ostia in the aortic wall, heavy enterostomy clamps on the auncles cut off much flow through this tissue prevented leaks but were so placed as not to interfere with the coronary venous return. Good checks were usually obtained at 160 and 100 mm Hg after initial perfusion with one liter at 200 mm. In the heart more often than in the kidney the second pair of observations indicated no change or slight slowing of flow rather than slightly faster flow. The arterial bed was visualized by roentgenograms after injecting lead gelatin at 40°C chilling and opening by Schlesinger's method (3). Perfusion and injection of lead gelatin increased the weight of the heart from the weights before and after study of intact hearts and before and after removing the auncles and great vessels it was found that the ventricular weight equalled 65 to 74 per cent of the final heart weight. The calculated ventricular weights of most hearts were taken as 70 per cent of the final heart weights.

As in the case of the kidneys, the results were all calculated on the conventional assumption that the pressure at the entrance to the coronary ostia was the same as that in the pressure bottle. Since the perfusion system always offers some resistance this assumption which seems to be universal in studies of organ perfusion introduces

a systematic error which is largest in cases with rapid flow and thus tends to increase the spread between high and low rates. In the heart perfusions a U tube connected to the two cannulae, and the system, without cannulae or other resistance gave a rate of flow, at 100 mm Hg of 3200 cc per minute, resistance ($R = P/F$) of 0.031. In a small heart, with total flow 490 and flow per gm of 2.9 cc per minute, recalculation after subtracting R of the perfusion system from observed R works out thus $0.204 - 0.031 = 0.173$, ideal flow = $\frac{100}{0.173} = 580$ cc per minute, flow per gm = 3.4

The usual calculation gives an error of -18 per cent. With a very fast flow and large heart, where total flow was 910 cc, flow per gm, 1.5 cc, the corrected figures are 1260 and 2.1 cc. Here the error is -40 per cent. It will be apparent from these examples that the figures for flow, calculated and recorded below in the conventional way, are too low by 20 to 40 per cent if one wishes to calculate the true resistance or flow capacity of the vascular bed of an organ. The uncorrected figures only are given, in order to keep the results comparable to those of other workers and in other organs. The only previous report on coronary perfusion gives no figures for heart weight (4) or flow per gram, but the total rates for flow of serum through the hearts of adults dying of infectious disease appear to be about one-fourth as great as those now reported.

RESULTS

In addition to the observations on normal hearts and hypertrophied hearts without gross narrowing of coronary arteries, these other data were obtained —

Normal young dog, flow at 100 mm Hg, 8.1 cc per gm per minute in a heart weighing 40 gm. Two old dogs, with marked renal hypertension and hypertrophied hearts, 125 gm heart, flow 3.75 cc per gm per minute, 140 gm heart 2.3 cc per gm per minute. Gollwitzer-Meier (5) reported blood flow rates up to 2 cc and Harrison (6) up to 5 cc per gm per minute in living dogs.

In infants, at 6 weeks 3.2 cc per gm per minute, at 18 months 5.2, and at 27 months 6 cc per gm per minute.

In two patients with narrowing but no occlusion of the coronaries 0.8 and 0.95 cc per gm per minute at 100 mm Hg. In another, with one main branch very narrow and two branches occluded, with large fibrous scars and small recent infarcts, 0.3 cc. per gm per minute.

The resistance to flow (Pressure/Flow) was greater at 100 mm than at 160. The rise in resistance at the lower pressure was 8.6 per cent in those under 60 years old, and slightly less, 6.5 per cent, in those between 60 and 89. There was no significant difference between those 40 to 60 and the younger group. Apparently the rigidity of the vessel walls is increased more in senility than in non-senile elderly persons. With perfectly rigid walls the resistance is the same at all pressures and the actual difference noted represents a 25 per cent increase in rigidity.

Other results are summarized in Figs 1 to 3.

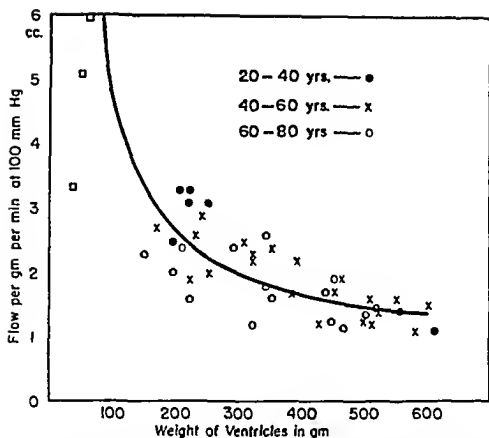


FIG 1 All data on kerosene perfusion of human hearts postmortem \square = infants 6 to 27 months, \bullet = adults of 20 to 40 years, \times = adults 40 to 60 years, \circ = adults 60 to 80 years. No cases with coronary narrowing demonstrable by x ray of heart after injection of lead gelatin are included. The heavy line, roughly relating flow to heart weight, has the formula $F = 0.7 + 400/W$.

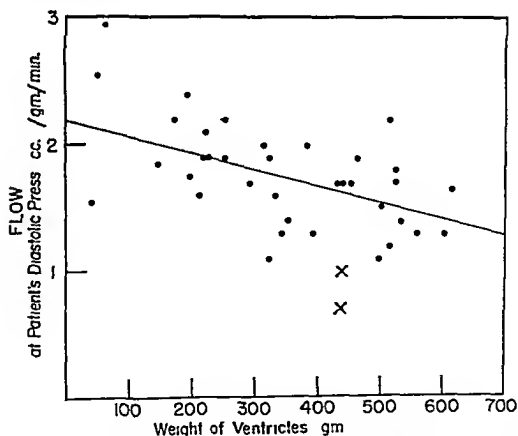


FIG 2 Kerosene perfusion flow at patient's diastolic pressure. This includes all observations on cases with repeated blood pressure observations for some time prior to terminal illness. The diastolic pressures of the three infants were arbitrarily taken as 55 mm Hg and in all cases diastolic flow calculated by multiplying flow at 100 mm Hg by diastolic pressure divided by 100. \times = cases of aortic insufficiency with low diastolic pressure.

Age—Age has a very striking effect on the vascular bed, even in hearts of the same weight and when all are free from coronary narrowing due to atheroma. In hearts of normal weight (ventricular weights of 150 to 250 gm) the

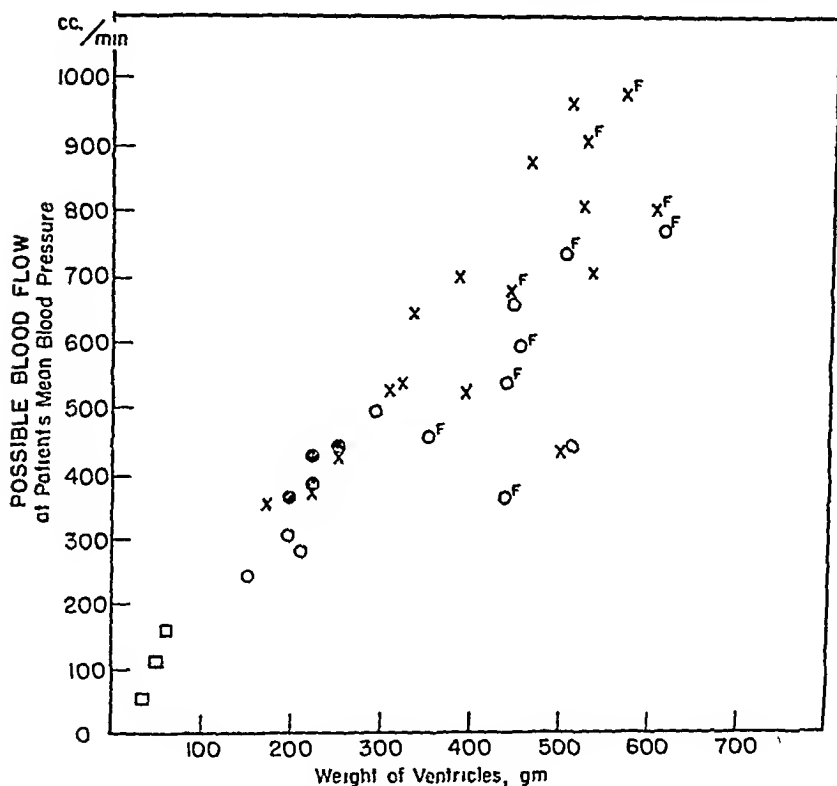


FIG 3 "Possible blood flow," correcting perfusion rate at 100 mm for patient's observed blood pressure (mean pressure taken as $S + D/2$), and for viscosity of whole blood compared with kerosene. \square = infants under 2½ years, \bullet = patients aged 25 to 40, \times = patients 40 to 57, \circ = patients 60 to 84 years old. An F to the right of a case indicates congestive failure. While flow at a constant level of pressure, or even at patients' diastolic pressure, falls with increase in weight of ventricles, the flow at mean pressure existing during life remains fairly constant in many cases in spite of hypertrophy. The maximal flow at mean pressure is given by the formula $F = 2 W + 50$, the minimal is $F = 0.9 W + 30$.

average flow at 100 mm Hg was 3.1 cc per gm per minute in those aged 25 to 40, 2.4 cc in those 40 to 57, and 2 cc in those 60 to 84 years old. There is a fall of 35 per cent in the average vascularity of hearts between the group 25 to 40 years and those over 60 years. This age difference is less striking with ventricular weight 260 to 400 grams, the average falling from 2.2 cc in those 40 to 60 to 1.9 in those 60 to 78 years old, and there was no significant difference

between these age groups in hearts whose ventricles weighed over 400 grams. The age effect in normal organs is not peculiar to the heart, for kidneys of people in the age group 45 to 60 have a perfusibility 32 per cent less than those from people 18 to 32 years old, and diodrast clearances indicate a similar decrease in actual renal blood flow with age (1).

The most striking change occurs with normal growth, and with change in age from 2 years to 30. Here the decrease in perfusibility of normal hearts is nearly 50 per cent, and this can scarcely be regarded either as a pathological or involutional process. It shows that decrease in perfusibility with age or increase in bulk may be a normal adaptive process.

Hypertrophy—The perfusibility of the heart decreases with hypertrophy. In the age group 40 to 60 the decrease is from 2.4 cc per gm per minute at weights under 250 gm, to 2.2 (—8 per cent) at weights of 250 to 400 gm, and to 1.5 (—37 per cent) at weights over 400. In the group over 60 years old, the decrease is from 2.0 cc with less than 250 gm of ventricle to 1.9 (—5 per cent) in those with 260 to 400 gm, and to 1.4 (—30 per cent) in those with over 400 gm of ventricular muscle.

Congestive Failure—There were four cases of aortic insufficiency in the group of heaviest hearts, two were rheumatic with hypertension and diastolic pressures of 80 mm Hg. All of them had congestive failure, and two had coronary flows, calculated for their diastolic pressure levels which were lower than any of the whole group, similarly reckoned. Otherwise there was no correlation, in a group of the same age and ventricular weight, between coronary flow and congestive failure (total eleven cases). Those dying of cerebral accident, prostatic disease, or other intercurrent illness had rates as low as or lower than any of the cases with congestive failure (Fig. 3). Nor did any of these nine cases show the fatty degeneration of the subendocardial fibers which is so characteristic of patients dying with severe coronary occlusion or acute or chronic anemia. In the latter group anoxia undoubtedly has been present for many hours before death, and is believed to be the cause of fatty degeneration. Such degeneration is rare in cardiac hypertrophy with no anemia or coronary disease.

DISCUSSION

As will be seen in Figs. 1 to 3 there is a great variation in coronary flow of hearts of the same weight, some of which is due to difference in age of the patient. The flow per gram of ventricular muscle, at the patient's mean or at his diastolic pressure, is much less variable over a wide range of heart weights than is the flow at a constant pressure level. Nevertheless age and hypertrophy do have a very striking effect in diminishing the capacity of the coronary bed. On the average a young adult with a normal heart has 2.4 times as wide a coronary bed as a man over 60 with a heart weighing 700 grams.

It is of course an everyday observation that young people with hyper

thyroidism, hypertension, or healed valvular lesions are much less apt to decompensate than are those with similar, or milder, cardiac burdens at ages over 45. It is equally certain that cardiac hypertrophy is invariably present and often is marked in those who have had weeks or months of congestive heart failure before dying. Is it then proper to conclude that the diminished coronary bed, per gram of muscle, which occurs with age and with hypertrophy is the main cause or even an important cause of progressive myocardial failure?

There are several precedents for ascribing heart failure to myocardial ischemia. Harrison (7) inferred as much from looking at the heart fibers and calculating that oxygen could not diffuse into their depths, and Wearn reached that conclusion after finding few capillaries per gram (2). Kountz, who measured the coronary flow in revived human hearts (8), also felt that the flow was regularly diminished after failure and that this was an important element in causing failure. Since he did not perfuse all the hearts at constant pressure, but began at 20 mm Hg and raised the pressure until dilatation set in, his published data do not prove that the coronary bed was narrowed, but only that the flow was poor at the levels of pressure which the muscle could maintain. In normal hearts he found the flow, at about 100 mm Hg, ran as high as 1.5 cc per gm per minute and that histamine doubled the flow, thus reaching a level only a little below that found on perfusing hearts postmortem with kerosene, which is half as viscous as blood. Kountz' results give us further confidence in the value of our method, but neither they, nor the morphologic studies of Wearn or Harrison prove that myocardial ischemia is the main or most important cause of heart failure, nor do the observations here recorded necessarily establish such a relationship. It is evident that when calculated for possible blood flow at the patient's mean blood pressure there is an increase in flow, in the majority of hearts, proportional to the increase in weight. The vascular bed is therefore apparently adapted to supply a constant *maximal* volume at the existing pressure.

Against the view that hypertrophy leads to ischemia are several observations which pathologists have often confirmed. Failure may occur with heart weights from 400 to 1100 gm, it may be absent in patients who die with hearts over 700 gm in weight. There certainly is no specific fiber thickness or level of hypertrophy which can be stated to be incompatible with an active life, with considerable capacity for muscular effort and increased cardiac work. It therefore seems unlikely that there is any fiber thickness or level of hypertrophy at which the heart becomes unable to carry a basal load because of anoxia, and until it fails under a basal load there is no fatal outcome. In the second place the pathologist regularly finds specific changes, notably subendocardial fatty degeneration, in hearts of patients with hemoglobin levels below 25 per cent, whether this is due to bleeding peptic ulcer or any other cause. These changes are not found in hypertrophy and heart failure. It is probable

that the fall of 60 per cent in coronary capacity for flow, found in the man of 65 with a 700 gm heart as compared with the man of 30 with a 250 gm heart, is less serious in its effect than a fall of 60 per cent in hemoglobin, for the latter causes an increase in cardiac output and work of the heart. Ischemia in either case may contribute to or aggravate heart failure, but it cannot safely be regarded as the main or most important factor. In this series of cases the capacity of the coronary bed at a constant level of pressure, was not less in those hearts which had manifested severe congestive failure than in the others, of the same weight, in which that phenomenon had not occurred, or was minimal and terminal.

It will be seen that the minimal rate of kerosene flow, calculated either at 100 mm Hg or at the patient's diastolic pressure, does not fall below 1 cc per gm per minute, except in aortic insufficiency. If the correction for difference in viscosity which we found to apply in the kidney is used for the heart, this would imply that the capacity for flow of whole blood never falls below 0.7 cc per gm per minute. Even in the innermost fibers of the left ventricle, where only diastolic flow may be possible, this would leave a capacity for flow of about 0.4 cc per gm per minute, carrying oxygen at the rate of 0.08 cc per gm per minute. The basal O₂ uptake of the human heart can be estimated from that of the dog—it is probably of the order of 0.03 to 0.05 cc per gm per minute. These calculations of flow are based on observed rates, uncorrected for slowing due to the resistance in the perfusion system and are at least 25 per cent below the true rate for possible blood flow. Hence, using the lowest possible values, in the least vascular hearts without gross coronary disease, the margin of safety is well above what is needed. In the great bulk of muscle, where flow continues through systole, and in the average heart at its usual mean pressure, even with the most marked hypertrophy there remains a capacity for coronary flow three to seven times as great as needed to supply oxygen, and sufficient therefore to maintain a fairly high O₂ tension about the muscle fibers.

It is of interest that while myohemoglobin in the heart increases in animals exposed to low O₂ tension it does not increase with hypertrophy (2 b). Hence the theory that myocardial ischemia is invariably present in cardiac hypertrophy involves an unverified assumption not only that cardiac vascularity fails to adapt itself to tissue needs in the usual way, but also that the normal adaptation of myohemoglobin to oxygen tension is inhibited.

The calculations of Harrison, which he uses to support the theory that diffusion of O₂ cannot take place into fibers as thick as those of the hypertrophied heart, are based on the assumption that the heart contains no oxygen buffer and the muscle contains no blood during systole, that the venous O₂ level is usually as low as that he observed in one series of dogs and much lower than he himself has recorded in hearts of dogs under maximal loads, and finally

that O_2 diffusion into a muscle fiber can be calculated with the formula for diffusion from a single tube in the center of a cylinder of tissue. Actually one must use the formula for a cylinder bathed in fluid with the same O_2 tension as mixed venous blood, and assume that O_2 diffusion takes place all through the cycle, since the small vessels always contain red cells and the muscle contains myohemoglobin. The O_2 tension probably does not drop to 0 during systole even deep in the left ventricle. If it did the venous sample would represent a mixture of entirely unsaturated blood from early diastole and highly saturated blood from the end of diastole, so that saturation of myohemoglobin and red cells in the capillaries would be very high at the onset of systole. Harrison himself noted coronary venous oxygen saturation of 40 to 50 per cent in hearts doing violent work, and equally high levels are found in the later, more extensive, and less indirect measurements of Visscher and of Gollwitzer-Meier. The possibility of high values is also indicated by these studies of the coronary bed.

If we use the formula of Hill (9, p. 60, *b*, 1) for diffusion into a cylinder of tissue using oxygen at a constant rate, and accept 0.12 cc O_2 per gm per minute as the highest possible rate of human heart muscle metabolism, it appears that fibers up to 60 microns thick will be supplied with O_2 even at their very center. Oxygen utilization at 0.07 cc per gm per minute is about twice the basal level and a coronary flow of 0.6 cc per gm per minute is easily possible. This would result in a venous saturation of about 35 per cent and a fiber 90 microns thick would be fully oxygenated. Since 16 to 18 microns is about normal, and 40 microns rarely is reached in the most hypertrophied hearts, it seems unlikely that hearts with such coronary beds as those here reported, and with normal blood hemoglobin, ever suffer from failure of O_2 to reach the centers of fibers. This is in accordance with the observations on nuclei, which lie in the center of these fibers and appear to be quite normal even in the most massive hypertrophy. Anemia does lead to nuclear as well as sarcoplasmic degeneration.

The clinicians who have accepted myocardial ischemia following hypertrophy as the explanation of congestive failure are welcome to see in this study a functional confirmation of views based on morphologic evidence. Certainly a 58 per cent decrease in capacity for flow is more startling than a 40 per cent decrease in capillaries per c mm. Most of this decrease was due to age alone, the greatest decrease in one age group was 37 per cent. It must be noted that the rates of flow in hypertrophy due to disease fall on precisely the same lines (Figs. 1 and 3) as those due to normal growth, and that a 46 per cent decrease in capacity for flow per gm of heart occurs between infancy and the prime of life. Recalling how the vascular bed, even in the aged, hypertrophies to supply a tumor, or to provide collateral about an occluded vessel, others may be

dissatisfied with a theory of myocardial failure based on the assumption that the vascular bed of the heart cannot adapt itself fully to supply the needs of the muscle

The pathologist or biologist perhaps can accept the thesis that the hypertrophied heart has a vascular bed ample for its needs, and that its fibers can be fully supplied with oxygen. They may not insist that the growth of the vascular bed exactly parallels an organ's increase in bulk. The pathologist may even be pleased that he need not explain to students why anoxia of heart muscle due to severe anemia or to coronary occlusion causes fatty degeneration, while the alleged anoxia of hypertrophy rarely does. Perhaps those of us who ascribe angina to myocardial ischemia will now find it easier to explain why angina is far less frequent with simple hypertrophy than with anemia and coronary disease. This may compensate for losing an explanation of myocardial failure which raised more questions than it answered.

SUMMARY

After eliminating vascular rigor, perfusing human hearts with kerosene under pressure postmortem gives values for coronary flow which seem an index of the maximum possible flow during life. This is 3.1 cc per gm per minute at 100 mm Hg in normal men under 40. It is 35 per cent lower in the hearts of those 60 to 80 years old, and also falls in hypertrophied hearts. In old people it is 30 per cent lower in hearts over 600 gm than in those under 350 gm; in patients 40 to 60 years old it is 37 per cent less in hearts over 600 gm as compared with those under 350 gm.

In discussion it is brought out that while the decrease in coronary capacity associated with age or hypertrophy may play a part in predisposing some hearts to congestive failure, there is no evidence that the hypertrophied heart has an inadequate oxygen supply or that its fibers are too thick for adequate oxygen diffusion. Congestive failure cannot be ascribed to anoxia except in the presence of severe anemia, coronary occlusion, or tachycardia with low blood pressure. Decrease in perfusibility with age and growth may be a perfectly normal adaptation to the needs of the tissue; the perfusibility of the heart of the young adult is about half that of an infant at 2 years.

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MYOHEMOGLOBINURIA

A STUDY OF THE RENAL CLEARANCE OF MYOHEMOGLOBIN IN DOGS*

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The observation that hemoglobin extracted from muscle was excreted by the kidneys much more rapidly than blood hemoglobin was perhaps the first indication that these two substances are different (4, 7). Since the purification and crystallization of myohemoglobin by Theorell (13) in 1932 many of its chemical and physical properties have been elucidated. Also ideas concerning renal function have become more specific so that many of the features which were so puzzling to early investigators have now become fairly obvious.

Camus and Pagniez (4) in 1902 were the first to report the experimental production of muscle hemoglobinuria. Their papers contain little specific information regarding experimental procedures, but they conclude significantly, that the amount of myohemoglobin recovered in the urine is proportional to the amount injected intravenously and that following such injections the urine becomes deeply colored while the plasma never exhibits more than a faint tinge. Similar results have been obtained in horses (3, 5) and the hemoglobinuria occurring in these animals in certain disease states has been attributed to the escape of myohemoglobin into the plasma and thence through the kidney. Whipple and his associates (1, 7, 10) also studied several aspects of the problem. Muscle hemoglobin was found to disappear rapidly from the plasma, and the renal threshold for this substance was estimated at about 15 mg. per kilo of body weight. Following repeated injections the iron content of the kidneys was somewhat increased although no iron-staining pigment could be demonstrated in the tubular epithelium, as in the case of blood hemoglobin.

The comparative physical and chemical characteristics of myohemoglobin and hemoglobin have been extensively dealt with in a recent review by Millikan (8). For the purposes of this study, however, the most significant findings are that the two substances are spectroscopically almost identical and that whereas hemoglobin has a molecular weight of 68,800 and contains four atoms of iron, a molecule of myohemoglobin weighs only 17,500 (12) and contains but one iron atom.

The experiments to be described are concerned with a study of the simultaneous renal clearances of myohemoglobin and creatinine in the dog. Since this method has previously been applied to hemoglobin (9), a quantitative

* We are indebted to Eli Lilly and Company for aid in conducting this work.

comparison of the renal excretion of the two substances can readily be made. The results show that myohemoglobin is cleared from the plasma about twenty-five times more rapidly than hemoglobin, but that the mode of excretion appears to be similar for the two substances.

Methods

All procedures employed for the determination of the renal clearances of myohemoglobin and creatinine were essentially similar to those described in a previous publication concerning the renal clearance of hemoglobin (9).

Female dogs were used to facilitate catheterization and tap water containing approximately 0.5 gm. of sodium bicarbonate per 100 cc. was given before and during each experiment in amounts sufficient to insure a moderate diuresis of alkaline urine.

A 10 per cent aqueous solution of creatinine was injected subcutaneously 1 hour before the commencement of each experiment in amounts approximating 1 cc. per kilo of body weight.

Urine was collected through a curved metal catheter, the bladder was washed with warm isotonic saline before and after each collection period, and the length of each period was approximately 20 to 30 minutes. The normal excretion rate of creatinine was determined during the first two periods and the myohemoglobin injections were made during the third. Following this, consecutive urine samples were collected until gross hemoglobinuria was no longer evident.

Blood samples of approximately 8 cc. were taken at about the mid-point of each period from the external jugular vein. In each instance, after puncturing the vein and withdrawing about 1 cc. of blood, a second syringe was inserted and the final sample collected. The blood was transferred with care to a tube containing 1.5 cc. of 1.4 per cent sodium oxalate and gently mixed. These precautions were necessary to prevent even very slight hemolysis which would interfere with the colorimetric determination of the relatively low plasma concentrations of myohemoglobin. Samples were centrifuged at high speed for 10 minutes and the plasma was transferred to another vessel immediately after noting the hematocrit. Appropriate factors were introduced into all final calculations to account for the anticoagulant.

Myohemoglobin was injected from a gravity bottle at a rate of from 20 to 30 cc. a minute. It was found desirable to inject as rapidly as possible, since myohemoglobin appeared in the bladder almost instantaneously, and the above mentioned speed was well tolerated.

In view of its extremely rapid elimination, the first urine period for myohemoglobin was measured from the time of completing the injection, and a blood sample was collected at the exact mid-point of this first period to avoid the necessity of interpolation.

Plasma and urine creatinine determinations were made according to the method described by Shannon, Jolliffe, and Smith (11). Myohemoglobin determinations were carried out according to the cyanmethemoglobin method of Evelyn and Salter (6). All colorimetric measurements were made on a Klett photoelectric colorimeter and appropriate blanks were carefully selected for each determination.

Preparation of Myohemoglobin—In order to obtain concentrations of myohemoglobin high enough to permit the rapid injection of a moderately large quantity, the

quantitative extraction method of Whipple (14) and the crystallization method of Theorell (13) were slightly modified and combined. Blood free muscles were obtained from dogs after viviperfusion, as described by Whipple (14). These were rendered reasonably free of fat and fascia, rinsed in saline, and minced in a meat grinder. Each kilo of ground muscle was mixed with about 1 liter of a 0.5 per cent solution of ammonia in water, shaken well and stored overnight at 2-4°C. The mixture was then filtered through several thicknesses of cheesecloth. The turbid reddish grey supernatant fluid was shaken in a separatory funnel with about one tenth of its volume of ether to remove any fat present and the relatively clear, red solution drained from the bottom was centrifuged. The pH was adjusted to 7.0 and basic lead acetate was added to precipitate a large portion of the protein impurities. The resulting heavy precipitate was removed by centrifugation, the pH again adjusted, and the excess lead removed by the addition of disodium phosphate. The final product, a clear, dark red fluid was dialyzed in cellophane sacs against distilled water at 2°C. Twelve hours were usually sufficient to remove all traces of phosphate. The solution was then rapidly frozen and lyophilized at reduced pressure and temperature. The brownish red flaky material so obtained was readily soluble in small volumes of distilled water (e.g. 300 mg per 10 cc. water). After filtration in a Büchner funnel it was ready for injection.

An early attempt was made to obtain a purer product by dialysis against saturated ammonium sulfate with subsequent removal of the sulfate by dialysis against water. However, after lyophilization a large proportion of the material was insoluble, indicating that denaturation had occurred. This procedure was abandoned because of the great loss of myohemoglobin, and because material obtained in this manner was found to be more toxic.

EXPERIMENTAL OBSERVATIONS

This report is concerned with the results of seven experiments performed on four separate animals. Varying amounts of myohemoglobin were injected intravenously, which caused initial plasma concentration ranging from 60 to 200 mg per 100 cc. Two animals received multiple injections but no appreciable difference was noted in the outcome of the various experiments. Small dogs were used exclusively in order to obtain maximum plasma concentrations with minimum amounts of myohemoglobin.

Slight reactions lasting $\frac{1}{2}$ to $\frac{3}{4}$ of an hour occurred following injection of the pigment in most experiments. The animals became listless and there was usually an associated diminution in the flow of urine and also in glomerular filtration, as estimated by creatinine clearance. These changes were attributed to the presence of some toxic impurities in the myohemoglobin preparations. In all other respects the clinical condition of the animals remained normal throughout.

Fig. 1 illustrates the rate at which myohemoglobin disappears from the plasma following intravenous injection. The composite graph was obtained by first plotting the disappearance curve for the experiment showing the highest

initial plasma concentration of myohemoglobin. In each of the other experiments the initial plasma concentration was placed on this curve and subsequent concentrations were plotted in relation to it with regard to time. It will be noted that the rate of removal from the plasma is very rapid above a concentration of about 30 mg per 100 cc, that it becomes much slower below this level,

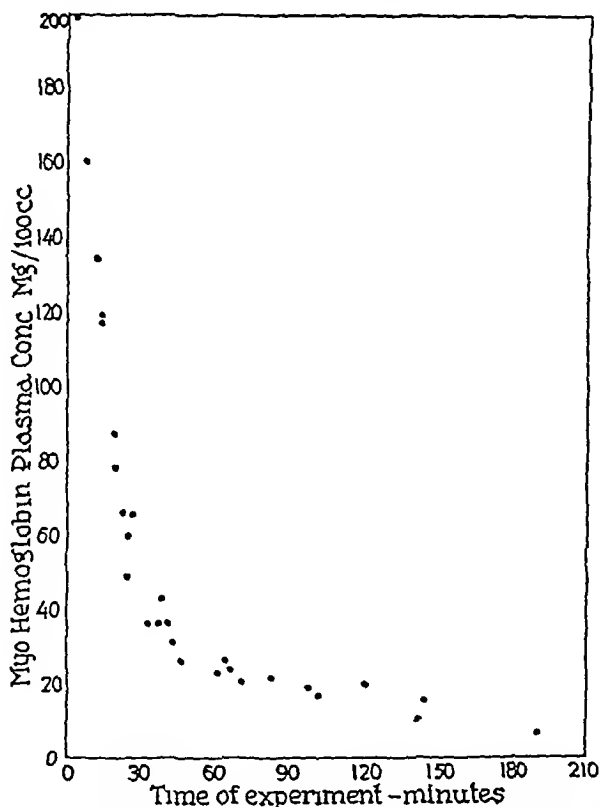


FIG 1 Composite graph showing the rate of disappearance of myohemoglobin from the plasma in relation to time, plotted on the basis of the curve obtained in the experiment showing the highest initial plasma concentration

and that within any given range of plasma concentration the disappearance rate is independent of the initial level instituted. Below a concentration of about 40 mg per 100 cc the plasma has only a very slight reddish tinge and tends to become somewhat yellow in color.

The detailed data obtained in one characteristic experiment are recorded in Table I. Columns 3, 4, and 5, respectively, list the average plasma concentration, the milligrams excreted per minute, and the clearance rate of creatinine in each separate period. Similar data pertaining to myohemoglobin are found in

columns 6, 7, and 8. The creatinine clearance rates (column 5) also designate the volume of fluid filtering through the glomeruli per minute in any given period. A temporary diminution in filtration is seen in period 3 immediately following the injection of myohemoglobin. The myohemoglobin/creatinine clearance ratios listed in column 9 are obtained by dividing the figures in column 8 by those in column 5, resolving any effects due to fluctuations in glomerular filtration. The values in column 10 represent the amounts of myohemoglobin recovered in the bladder urine in each period, listed as a percentage of the total amount injected, and column 11 indicates the relative rate of urine flow from period to period.

TABLE I
Summary of Experiment 1
Dog 40-65—Weight 10.5 Kilos

Period	Length of period	Creatinine			Myohemoglobin			Myohemoglobin/creatinine clearance ratio	Myohemoglobin in urine	Urine flow
		Plasma	Urine	Clearance	Plasma	Urine	Clearance			
1	2	3	4	5	6	7	8	9	10	11
—	min	mg/100 cc	mg/min	cc/min	mg/100 cc	mg/min	cc/min	—	per cent	cc/min
1	27 5	7 85	3 49	44 5	—	—	—	—	—	4 5
2	25 0	7 15	3 05	42 7	—	—	—	—	—	3 0
100 gm myohemoglobin dissolved in 75 cc water injected intravenously (95 mg/kg)										
3	17 5	6 60	2 22	33 6	94	20 00	21 0	0 59	34 5	0 6
4	22 0	5 98	2 50	41 8	38	7 72	20 3	0 49	17 0	1 3
5	27 5	5 45	2 47	45 1	26	2 94	11 3	0 25	8 1	3 1
6	30 5	4 80	2 23	46 5	21	1 28	6 1	0 13	3 9	3 6
7	62 0	3 94	1 81	46 0	18	0 33	1 83	0 04	2 0	2 0

In Fig 2 the excretion rates of myohemoglobin and creatinine in relation to their respective plasma concentrations are represented graphically. The creatinine curve shows a characteristic straight line originating at zero while a straight line relationship between the plasma concentration of myohemoglobin and the milligrams excreted per minute is seen to exist above a threshold plasma level of 17 mg per 100 cc. In both curves the excretion during period 3 is lowered. The disappearance of these variations in the myohemoglobin/creatinine clearance ratio indicates that the same mechanism, presumably lowered filtration, is responsible for the observed changes in the excretion of both substances. When the myohemoglobin value in this period is corrected for the percentage drop in creatinine excretion, it is found to fall on the straight line joining the other points. A definite straight line relationship between the plasma concentration and the rate of excretion of myohemoglobin is found in

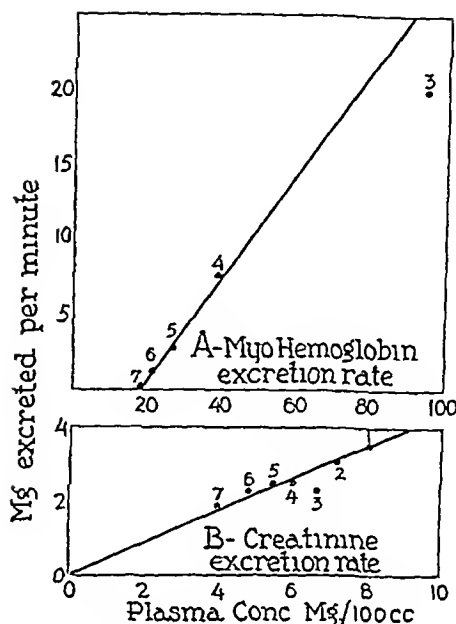


FIG 2 Graphs A and B show the relationship between the rate of renal excretion and the plasma concentration for myohemoglobin and creatinine respectively, in experiment 1

TABLE II
Summary of All Experiments

Ex- per- iment	Dog	Weight	Myohemoglobin injected		Renal thres- hold	Max myohemo- globin clear- ance		Average creatinine clearance	Max myo- hemoglobin/ creatinine clearance ratio	Per cent of injected myohemo- globin in urine
						Actual	Cor- rected			
1	2	3	4	5	6	7	8*	9	10	11
No	No	kg	gm	mg/kg	mg/100 cc	cc/min	cc/min	cc/min	—	—
1	40-65	10.5	1.00	95	17	18.8	24.4	43.6	0.59	68
2	40-65	11.8	1.15	98	18	12.3	24.2	42.5	0.57	63
3	40-65	11.9	0.90	75	17	14.8	26.4	45.2	0.61	67
4	39-104	9.1	0.78	85	20	20.9	26.6	44.7	0.53	63
5	39-104	9.1	1.50	167	18	15.0	23.0	40.0	0.56	65
6	39-242	8.0	0.66	82	23	32.5	32.5	59.0	0.55	63
7	40-370**	10.7	0.99	92	—	16.3	35.0	62.0	0.57	—

* Figures in column 8 are corrected for the percentage drop in creatinine clearance during the initial myohemoglobin period

** Dog 40-370—experiment discontinued due to trauma to urethra.

all experiments when similar corrections for variations in glomerular filtration are applied

Table II summarizes the salient features of all the experiments. Columns 3, 4, and 5 list the amounts of myohemoglobin injected and their relationship to body weight. It will be seen that the renal threshold values in column 6 show a striking relative uniformity. However, it is felt that these values may be a little too high due to the slight effect of the yellow color in the plasma on the colorimetric readings at low plasma concentrations. The possibility of some contamination of the myohemoglobin solutions with blood hemoglobin was

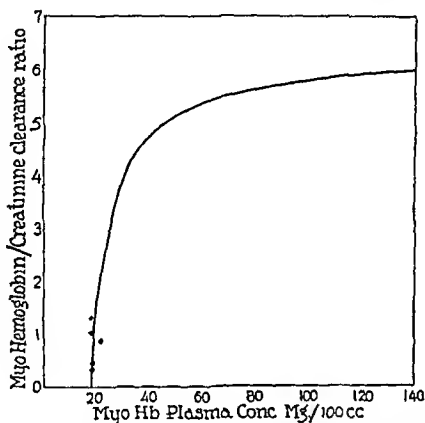


FIG. 3. A composite graph relating the myohemoglobin/creatinine clearance ratio to the plasma concentration of myohemoglobin in all experiments.

also considered. However, random histological sections of the muscle tissues used for extraction showed a complete absence of red blood cells after *vivi* perfusion. It is also significant that on two occasions when samples of pigment, excreted by the kidneys below the renal threshold for blood hemoglobin, were reinjected, after dialysis against water, the threshold values observed were identical with those originally obtained.

The figures in column 7 are the actually determined clearance rates obtained in each experiment during the first period following the injection of myohemoglobin. In all but one instance there was a drop in creatinine clearance during this initial period below the average levels listed in column 9. The values in column 8 represent the maximum myohemoglobin clearance, under

centrations (4) The estimated total amount of hemoglobin reabsorbed (Table II, column 9) is calculated from rates of tubular reabsorption so obtained, both above and below the threshold, and the time required to clear the plasma of hemoglobin. Graph D shows the excretion rate curves for dog 39-225, both initially and after repeated hemoglobin injections. Both curves are parallel originating at the respective threshold levels and it is apparent that a marked

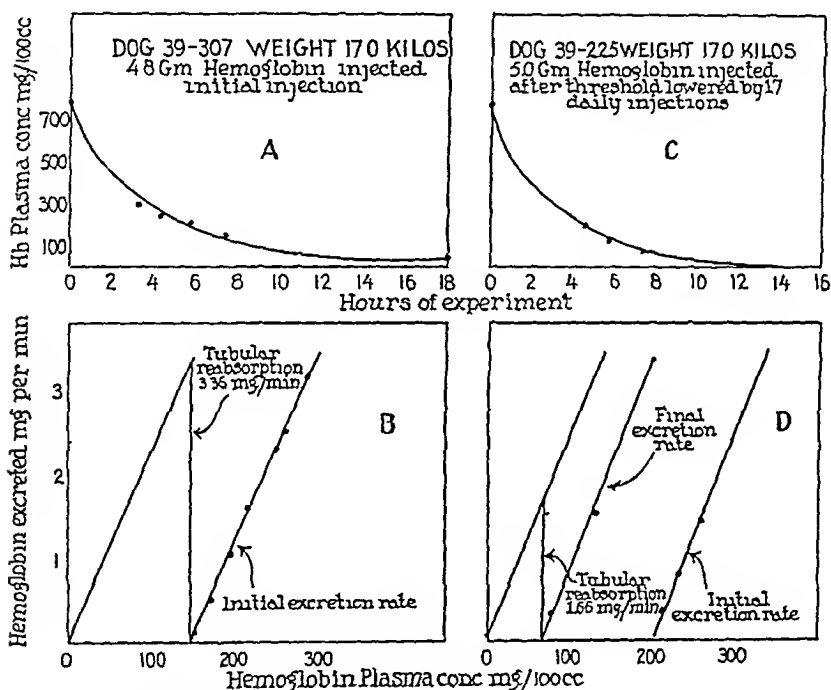


FIG 1 A graphic illustration of the results obtained following the injection of hemoglobin containing radio-active iron, in one normal dog and in one with a lowered threshold

Graphs A and C represent the rates of disappearance of hemoglobin from the plasma, and Graphs B and D show the curves relating the rates of renal excretion to plasma concentration

diminution in the theoretical reabsorption rate accompanies the lowering of the threshold

Histologically, there were dense iron-staining deposits of pigment in the epithelium of the convoluted tubules in the animals subjected to repeated hemoglobin injections but no traces of iron-staining pigment were seen in the normal group 24 hours after the initial injection. The hemoglobin stain employed showed a complete absence of red blood cells in the kidneys and in both groups of animals there was a delicate blue, finely granular haze visible in the

cytoplasm of the epithelium cells of the convoluted tubules. Iron staining pigment was not stained by this method.

The fact that no iron-staining material was observed after the initial injection of hemoglobin in the normal dogs indicates that the pigment remaining in the kidneys is not markedly altered within 24 hours.

DISCUSSION

The earlier observations on the lowering of renal thresholds for hemoglobin by repeated injections have been confirmed and variations in the threshold have been related to plasma concentration. From the foregoing experimental data, it is apparent that this phenomenon is not associated with a cessation of tubular reabsorption as previously suggested by Newman and Whipple (5). Since in the early experiments *diminishing* doses of hemoglobin were given, it is possible that the relatively constant low threshold levels attained were due to a lowered rate of tubular reabsorption representing a state of equilibrium between the amounts of hemoglobin picked up by and removed from the tubular epithelium each day.

If the concept of a constant rate of glomerular filtration and a rate of tubular reabsorption which reaches a maximum at the threshold (4) is correct, the parallelism between hemoglobin excretion rate curves, obtained initially and after lowering the threshold, indicates that a diminution in the rate of tubular reabsorption is responsible for the changes observed in the threshold level. Under these conditions it is reasonable to suppose that a cessation of tubular reabsorption would coincide with a complete elimination of the threshold. However, when hemoglobin containing radio active iron is injected, the kidneys of animals with lowered thresholds are found to have retained more hemoglobin products in 24 hours than the kidneys of *normal animals*.

As a tentative explanation to account for the discrepancy between the estimated total tubular reabsorption and the actual amount of radio-active iron retained by the kidneys, it is suggested that in normal animals hemoglobin is rapidly removed from the renal epithelium. This would imply that when the threshold has been lowered by repeated daily injections, the rates of both reabsorption from the tubular lumina and removal from the tubular epithelium are diminished, since under these conditions the estimated and actual amounts of hemoglobin picked up are approximately equal.

It is conceivable that the phenomenon results from some modification of glomerular permeability without alteration of the rate of tubular reabsorption, but definite proof one way or the other must await further investigations.

SUMMARY

A drop has been observed in the renal threshold for hemoglobin in dogs, of over 60 per cent, following repeated injections daily. It was not associated with a cessation of tubular reabsorption.

Hemoglobin excretion rate curves, obtained initially and after lowering the threshold, have proved to be parallel lines originating at the respective levels

Hemoglobin containing radio-active iron has been used to determine the amount of iron retained by the kidneys 24 hours after injection

The kidneys of normal animals retain slightly less iron than those of animals with lowered thresholds, despite the fact that the former group has a much higher estimated rate of tubular reabsorption

It is suggested that hemoglobin products are more rapidly removed from the kidneys of normal animals, following reabsorption, than from those of animals which have received multiple injections of hemoglobin

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THE BEHAVIOR OF POX VIRUSES IN THE RESPIRATORY TRACT

IV THE NASAL INSTILLATION OF FOWL POX VIRUS IN CHICKENS AND IN MICE

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PLATE 9

(Received for publication June 7, 1941)

The work which is here reported was undertaken in part as an extension of our previous observations on the behavior of pox viruses in the respiratory tract (1, 2) and in part to determine whether or not fowl pox virus was capable of simulating the clinical syndrome of chickens referred to as roup. Roup, which is also known by a variety of names as infectious catarrh, infectious rhinitis, and fowl coryza, is defined as a communicable disease of natural occurrence in chickens characterized by nasal and ocular inflammation which may or may not be accompanied by an outward discharge.

Even before the etiology of roup was established a number of investigators, Haring and Kofoid (3), Beach (4), Doyle and Minett (5), were agreed that it was a distinct entity and unrelated to fowl pox. Their evidence was incomplete but was nevertheless significant since reciprocal protection was not demonstrable. We began an investigation of the syndrome in 1932 shortly after De Bieck (6) had shown that the influenza like bacterium now known as *Hemophilus gallinarum* was of etiological significance. His work and ours (7-9), which led to the recognition of three clinical types of the disease, were likewise in agreement with these conclusions and afforded ample evidence that roup may occur naturally and be reproduced experimentally in the absence of fowl pox. Much of the earlier work was concerned with the skin inoculation of normal birds with nasal exudates from naturally infected chickens, fowl pox virus not infrequently being demonstrable. The actual nasal instillation of virus suspensions or of exudates was only occasionally undertaken, with scant reference to the results even though an ocular nasal form of fowl pox was recognized. Our observations as outlined in this paper are largely concerned with this route of introduction, nasal injections of fowl pox virus being made in the absence of the causative agents of roup.

Source and Activity of the Virus

The strain of virus used in most of the following experiments was one obtained locally in 1937 from a field case of fowl pox and maintained thereafter by occasional skin passage in susceptible birds

Saline suspensions of crusts or of the contents of vesicles produced typical pox when rubbed into the follicles of the leg after removal of the feathers. This method was always used for testing the activity of the virus in the skin, 3 to 6 months old Rhode Island Red, Barred Rock, or cross-bred birds being employed. Enlargement of the follicles was generally observed on the 3rd day but was occasionally delayed until the 4th day after inoculation. The reaction progressed rapidly thereafter, reaching its height on about the 7th day when the follicles were markedly nodular, often reddened at the base, and capped by a dirty white tip. Retrogressive changes set in quickly and scabbing frequently occurred without apparent vesiculation. The area of inoculation covered 50 to 75 feather follicles all of which were generally involved. At the height of the reaction the intervening skin between the follicles was sometimes thickened and on removal showed marked subcutaneous edema. Later the entire area was often covered by a thick reddish brown crust. Inoculated birds rarely showed any obvious generalized response to the virus.

In sections of the skin stained with phloxin-methylene blue there was an initial hyperplasia of the follicular epithelium followed by necrosis and a widespread formation of cell inclusions. Most sections also showed a marked infiltration of lymphocytic cells around the follicles and also in the deeper layers of the skin. In films of vesicular fluid impregnated with silver by the Morosow method elementary bodies were usually demonstrable in the roughly circular groupings which appear to be particularly characteristic of fowl pox.

The activity of the Princeton virus was compared with that of a known strain of fowl pox which was kindly supplied by Dr. F. R. Beaudette of the New Jersey Agricultural Experiment Station in New Brunswick, N. J. This virus had been carried through 49 transfers in embryonated eggs. Each of the two fowl pox strains was tested by follicular inoculation in 3 chickens, with complete reciprocal protection. There was also a reasonably close parallel, both qualitatively and quantitatively, in the reactions produced by them. Elementary bodies appeared to be somewhat more numerous in films from chickens inoculated with the Beaudette strain.

Nasal Instillation of the Virus Propagated by Skin Passage

Reaction in the Nasal Passages—The results of a preliminary experiment in which 8 chickens were injected intranasally with a follicular suspension of the Princeton fowl pox strain indicated survival for at least 15 days. Although elementary bodies and specific mucosal changes characterized by epithelial hyperplasia and inclusion formation were demonstrable, these findings were irreg-

ular and the only certain test for the presence of virus was the cutaneous inoculation of a susceptible chicken with nasal washings

The suspension used in this experiment was prepared from the 7th follicular passage of the virus. The contents of about 15 pox were removed on the 7th day after inoculation and pooled in 5 cc of saline. Small unmeasured amounts of the suspension were introduced with a capillary pipette in the nasal passages of 8 birds by way of the palatine cleft. The chickens used for nasal instillation were 2 to 3 months old and were usually younger than those employed for follicular inoculation. The infected birds were held under quarantine in separate cages and killed in pairs on the 10th, 11th, 14th, and 15th days. At autopsy 5 of them showed some thickening of the nasal mucosa but in only 2, killed on the 10th day, were pock like nodules observed. A thick mucocellular material was aspirated from the nasal passages with a pipette but there was little if any increase in volume over that removable from uninfected birds. The sinuses, larynx, trachea, pharynx, and eyes were normal in all of the chickens and there was no discharge from the nares. There were no symptoms of nasal irritation or of a generalized response to the virus nor any indication of a subsequent involvement of the skin.

Elementary bodies were demonstrable in silver impregnated films of nasal washings from 6 of the infected chickens (Fig 1). Sections from 4 of them showed epithelial hyperplasia and inclusion formation (Fig 2). Nasal washings from 2 killed on the 10th and the 15th day, produced a typical skin reaction on follicular inoculation in susceptible birds.

Transmission by Nasal Passage—Following establishment of the virus in the upper respiratory tract of a susceptible chicken it was maintained for 12 successive nasal passages at intervals of 10 to 11 days. In the last 7 passages the suspensions used for nasal instillation were also tested by skin inoculation, all of them containing sufficient virus to produce a typical follicular reaction.

The initial suspension was made from the 8th skin passage of the virus. The subsequent inocula were prepared by suspending nasal scrapings or the catarrhal material removed on aspiration in 2 cc. of saline. The passages were carried out at intervals of 10 to 11 days, injection being by way of the palatine cleft. 7 of the infected birds showed visible thickening of the nasal mucosa accompanied in one instance by erosion of the epithelial lining of the turbinates. Pox like lesions were not encountered but epithelial hyperplasia with inclusion formation was observed in sections of the nasal mucous membrane from 4 birds. Sections from 3 showed some edema and cellular infiltration. The eyes, sinuses and tracheas were again normal and there was no discharge from the nares. Elementary bodies were demonstrable in silver impregnated films of nasal washings from 10 of the 12 chickens. The extent of the skin reaction in the birds which received a follicular inoculation of the nasal suspensions was indicative of a moderate titer of virus, the number of involved follicles varying from 25 to 60.

Transmission by Direct Contact—The communicability of the nasally established virus was determined by contact experiments in which a susceptible bird

was placed in the same cage with one previously infected by nasal instillation. Development of the virus in the upper air passages of the initially infected birds, 9 in number, was indicated by direct microscopic examination or by subsequent protection tests. There was no demonstrable transfer of the virus from these birds to those in direct contact with them.

Three groups of chickens were used in this experiment. 2 of the initially infected birds were injected intranasally with nasal washings from passage 10 of the transmission series. 3 were similarly injected with nasal washings from passage 11 of the same series, and 3 with a suspension prepared from the 13th skin passage. The susceptible chickens in Group 1 were placed in contact with the infected ones on the 2nd day after nasal instillation, those in Group 2 on the 4th day, and those in Group 3 on the 5th day. The exposed birds in Group 1 were killed on the 9th day of contact, while those in Groups 2 and 3 were killed on the 14th or 15th day. All of them remained normal during the period of exposure. At autopsy the nasal passages of the exposed birds showed no apparent involvement, nor were elementary bodies demonstrable in silver impregnated films. The feather follicles of susceptible birds inoculated with nasal washings from each contact chicken showed no reaction during a period of 2 weeks.

Protection against Follicular Inoculation—Nasal instillation of the virus maintained by dermal passage or by nasal passage afforded complete protection to a subsequent follicular inoculation. None of the 9 birds in this series showed any local reaction when fowl pox virus was introduced into the skin 4 to 6 weeks after the initial nasal instillation.

7 of the infected birds from the contact series and 2 additional ones were used in this experiment. 3 were injected intranasally with nasal washings from passage 11 of the transmission series and were tested 47 days later by follicular inoculation with a suspension made from the 13th skin passage. A susceptible chicken was similarly inoculated at the same time. 6 of the birds were injected intranasally with the 13th skin passage suspension and were tested, together with a susceptible chicken, 31 days later with virus from the 14th skin passage. The 9 birds which had received the initial nasal instillation were held under observation in separate cages and examined daily for at least 2 weeks. None of them showed any involvement of the inoculated area during the period of examination. In the 2 susceptible birds there was a typical local reaction which began on the 3rd day and covered a wide area of skin.

Nasal Instillation of the Virus Propagated by

Transfer in Embryonated Eggs

Reaction of the Virus in the Nasal Passages and Its Transmission—The Princeton strain of fowl pox was established in the nasal passages of susceptible chickens following nasal instillation of membrane suspensions from the 6th, 34th, and 50th transfers in embryonated eggs and was subsequently main-

tained for 5 passages by the injection of nasal washings. In 10 of the 15 birds used in this series there was no apparent mucosal reaction, elementary bodies were not detectable, and the presence of virus was demonstrated only by the follicular inoculation of susceptible birds. The concentration of virus in the nasal washings as indicated by the number of follicles involved on subinoculation tended to be low.

The virus was isolated in a pure state, free of bacteria, and maintained for 50 transfers by inoculation of the chorioallantoic membrane of 10 day embryonated hen's eggs. Prior to inoculation the membrane was retracted by the Burnet method. Approximately 10 per cent suspensions in saline were prepared by grinding the inoculated membranes after incubation of the eggs for 3 to 4 days at 37°C. At this time the chorioallantois showed a wide, confluent or semiconfluent, central area of hyperplasia. Typical fowl pox inclusions were demonstrable in sections and elementary bodies in silver impregnated films. The embryo was usually alive and active.

2 chickens were inoculated, one *via* the palatine cleft and one by way of the feather follicles, with suspensions of the 6th, 34th and 50th egg transfers of the virus. The 3 birds which received the follicular inoculation showed a typical fowl pox reaction which began on the 3rd day and involved 50 to 75 follicles. The virus which was established by nasal instillation was subsequently maintained for 5 passages by injecting a saline suspension of nasal washings at intervals of 10 or 11 days. With each passage the follicles of a susceptible bird were inoculated with the same suspension used for the following nasal instillation. One bird in each of the 3 passage series showed macroscopic involvement of the nasal mucosa. Elementary bodies were demonstrable in nasal films from one bird in each of the first 2 series and from 2 birds of the 3rd series. In 8 of the birds inoculated dermally with nasal suspensions the reaction approximated that with skin or egg membrane suspensions. 40 to 60 follicles being involved. In 7 of these birds there was a reduced reaction involving only 10 to 30 follicles.

Communicability and Protective Action of the Virus—Susceptible chickens placed in direct contact with birds infected by nasal instillation of the 34th egg membrane transfer and exposed to them for approximately 2 weeks showed no resulting skin lesions nor was virus demonstrable in the nasal tract either by direct examination or by follicular inoculation. A sufficient degree of immunity to inhibit development of the virus in the feather follicles was not afforded by nasal instillation of the egg membrane suspension. The reaction which attended follicular inoculation of the initially infected birds, after a period of 6 weeks, was however atypical and indicative of partial protection.

The 34th egg membrane suspension used in these inoculations was made at a later date than that in the preceding experiment. Rubbed into the feather follicles of a susceptible chicken it produced a typical reaction over a wide area. There was no apparent involvement of the local mucosa on nasal instillation nor were elementary bodies demonstrable in films. Development of the virus was indicated, however by

a follicular reaction following the inoculation of nasal washings. The titer of virus in the nasal suspension was probably low as only 20 follicles were involved.

A susceptible chicken was placed in the same cage with each of 3 birds on the 4th day after nasal instillation of the egg membrane suspension. Contact was maintained through the 16th day when the exposed birds were removed and killed. At autopsy there were no skin lesions, the nasal mucosa appeared normal, elementary bodies were absent, and nasal washings were inactive on follicular inoculation in susceptible birds.

The initially infected birds were held for 31 days and tested by dermal inoculation with a suspension of the 12th skin passage of the virus. Each of the 3 birds showed swelling of the follicles on the 3rd day but the reaction did not progress to the formation of typical pox. The follicles were definitely nodular by the 5th day, as many as 40 being involved, but there was neither congestion nor vesiculation. The follicles remained nodular without scab formation through the 13th day when the chickens were killed.

TABLE I

The Survival of Fowl Pox Virus in the Lung of the Mouse Following Nasal Instillation

No. of days to autopsy	No. of mice	No. of mice with lung lesions	No. of virus recoveries from lung
3	14	14	14
5	12	7	5
7	11	8	4
10	8	7	0

Nasal Instillation of the Virus in Mice

Mice injected intranasally with egg membrane suspensions of fowl pox showed no symptoms indicative of either a general or a local development of the virus. At autopsy the mucous membrane of the upper respiratory tract appeared normal, there was no exudate in the nasal passages, and elementary bodies were not detectable in nasal washings. The specific virus was regularly recoverable, however, from the lung through the 3rd day after nasal instillation and irregularly through the 7th day. Its presence was generally attended by a slight but readily recognized pulmonary reaction. The number of virus recoveries and of involved lungs at intervals of 3, 5, 7, and 10 days is presented in Table I.

Etherized mice weighing 15 to 20 gm. were infected with the Princeton strain of fowl pox virus by inhalation, the nose being held for 8 or 10 respirations in a Petri dish containing a saline suspension of the virus. Approximately 10 per cent suspensions of bacteriologically sterile membranes removed from embryonated eggs on the 3rd or 4th day after inoculation were employed. They included the 6th, 12th, 34th, and 50th egg transfers. The infected mice were killed on the 3rd to 10th day, the nasal passages were exposed, and from some of them silver impregnated films were made of material aspirated with a capillary pipette. The lungs were removed and examined microscopically at a magnification of around 10 diameters. They were then

ground with a little saline after removal in some cases of a small piece for sectioning, in a glass tissue grinder and suspended in several cubic centimeters of the same diluent

All of the infected mice were normal in appearance when brought to autopsy, their coats were smooth, and those killed on the 7th day or later had generally gained in weight. The only pathological change encountered at autopsy was a slight reaction in the lung which was best observed under a dissecting microscope at low magnification. It resembled the reaction previously described in the lungs of mice infected intranasally with variola virus (2). The lungs showed single or multiple patchy areas of solidification, grayish in color or tinged with pink, which contrasted sharply with the pale foamy structure of the normal pulmonary tissue. Histologically there was a marked increase in the number of mononuclear cells and lymphocytes around bronchioles and blood vessels and in discrete islands just beneath the pleural surface or centrally located (Fig 4). In a few sections the alveoli showed mononuclear cells and erythrocytes. Some of the bronchioles contained desquamated epithelial cells but there were no cell inclusions. The membranes of embryonated eggs inoculated with lung suspensions generally showed discrete foci varying from 5 to 100 or more, indicative of a low concentration of virus. The membrane from an egg inoculated with the suspension of a lung removed from a mouse on the 7th day after nasal instillation is shown in Fig 3. The subinoculation of membranes with discrete foci resulted in a typical confluent or semiconfluent reaction covering a wide area.

DISCUSSION

Fowl pox virus from active skin lesions was repeatedly established in the upper respiratory tract of normal chickens and recovered in mucosal washings for as long as 15 days after nasal instillation. Multiplication of the virus was clearly indicated by its maintenance for 12 successive host passages, the resulting dilution factor being much greater than the titer of the original inoculum. The experimentally infected birds showed no skin lesions and no external evidence of nasal or ocular involvement. The lack of conjunctival manifestations is in contrast to the findings recorded during a natural outbreak of uncomplicated fowl pox in which 20 per cent of 50 chickens with skin lesions also showed unmistakable signs of ocular inflammation.

A specific mucosal reaction was only irregularly observed at autopsy. Many of the infected birds showed no evidence of nasal involvement although fowl pox virus was readily demonstrable. Indeed the only certain test for the presence of virus was the activity of nasal washings on subinoculation in the skin of a normal bird. The nasal passages sometimes contained a thick catarrhal material but frank exudation was rarely observed and the adjacent sinuses were invariably normal. The condition of the upper respiratory tract in the presence of fowl pox virus is very different from that in the presence of the causal agents of roup. Natural or experimental roup infection is frequently attended by a nasal discharge which with the coryza of slow onset may persist for weeks. At autopsy a copious mucopurulent or catarrhal exudate is almost invariably present in the nasal passages and the sinuses. Birds infected with

roup may or may not show ocular involvement, but if they do it occurs in conjunction with nasal inflammation and not as an independent manifestation

Fowl pox virus established in the upper air passages following nasal instillation was readily transmitted by the passage of nasal washings but it was not transmitted by direct contact. Development of the virus in the nasal mucosa is not attended by an outward discharge and presumably the amount of virus which escapes by way of the nares is too small to infect an exposed bird. The volume of inoculum used experimentally contained a relatively high concentration of virus. It is probable that the nasal tract as such is of little significance in the spread of fowl pox under natural conditions. It should be borne in mind, however, that in nature such factors as coexisting infections, unfavorable dietary and climatic conditions, and strain differences might influence the outward and inward nasal passage of the virus.

The nasal establishment of fowl pox virus was attended by protection of at least 4 to 6 weeks duration against development of virus in the skin on subsequent follicular inoculation. The number of birds tested was too small, however, to warrant any conclusions as to the constancy or duration of the protection afforded by nasal immunization. Doyle and Minett (5) had noted earlier that the presence of fowl pox virus in the nasal passages of naturally infected birds did not necessarily result in protection against cutaneous inoculation.

The preceding observations were from chickens injected intranasally with fowl pox virus from active skin lesions. Virus propagated for as many as 50 transfers in embryonated eggs was also established in the nasal tract and maintained for a sufficient number of passages to indicate multiplication. In general the activity of the virus after carriage in fertile eggs appeared to be somewhat less than that tested directly from the skin. Elementary bodies were rarely demonstrable in nasal films from infected birds and inclusion bodies were not observed in sections of the mucosa. Birds injected with the 34th egg transfer were not completely protected against development of the cutaneous strain on subsequent follicular inoculation. The number of birds tested was again too small to be certain whether the failure to afford complete protection was the result of some change brought about by transfer in the egg or was simply an expression of variability attendant on nasal immunization.

The behavior of the egg transfer strain of fowl pox virus on nasal instillation in mice resembled that of variola virus (2). There were no attendant symptoms and no apparent changes in the nasal mucosa. The specific virus was recoverable, however, from the lung through the 7th day, survival being accompanied by a slight pulmonary reaction.

SUMMARY

Fowl pox virus from active skin lesions was established in the upper respiratory tract of normal chickens by nasal instillation and maintained for 12

successive passages. The nasal infection was not communicable by direct contact but did afford protection, for at least 6 weeks, against subsequent development of the virus in the skin.

Multiplication of the virus in the nasal passages was only irregularly attended by specific mucosal changes and was not accompanied by the vigorous counter reaction engendered by the causal agents of roup.

The same strain of virus on propagation in embryonated eggs also survived and multiplied in the nasal tract but with somewhat reduced activity, the 34th egg transfer failing to afford complete protection. Nasal instillation in mice was followed only by a reaction in the lung from which the virus was recoverable through the 7th day.

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EXPLANATION OF PLATE 9

FIG 1 Elementary bodies in silver impregnated film of nasal washings from a chicken infected with fowl pox by nasal instillation $\times 1580$

FIG 2 Section of nasal mucosa showing epithelial hyperplasia and inclusion bodies from a similarly infected chicken Phloxin-methylene blue $\times 113$

FIG 3 Specific foci in the chorioallantois of an embryonated egg inoculated with lung suspension from a mouse killed 7 days after nasal instillation of fowl pox virus $\times 115$

FIG 4 Section of a lung showing cellular reaction in alveoli and around vessels from a similarly infected mouse Phloxin-methylene blue $\times 100$

The photographs were taken by Mr J A Carlile



(Nelson Pox viruses in the respiratory tract IV)

are parasitized At the 72 to 96 hour period a preponderance of the microorganisms is present within the cytoplasm of the endothelial cells lining the capillary plexus which directly underlies the ectoderm and also in those of many of the veins scattered throughout the mesoderm These infected cells are distended enormously and eventually burst The infection extends from cell to cell and in many instances the entire vessel wall is outlined with endothelial cells distended with microorganisms During the earlier passages in the membrane many of the mesodermal vessels exhibit a response to the presence of the bacilli which is characterized by endothelial cell proliferations which form papillary projections in the lumen at times causing complete occlusion This response in many instances is observed to be associated with the presence of a small number of bacilli within some of the endothelial cells Coincident with the invasion of mesodermal endothelium there is marked activity on the part of mononuclears and fibroblasts which form granulomatous nodules which are especially prominent about the blood vessels The centers of some of these granulomata undergo necrosis The microorganisms if present are seen within fibroblasts or mononuclears at the periphery of these lesions and not in the intercellular spaces or within the necrotic areas at their centers In areas where injury to the vessels has resulted in hemorrhage small numbers of bacilli multiply to some extent extracellularly but the preponderance of proliferation proceeds within cells of mesodermal derivation Inflammatory reaction is slight, consisting chiefly of large mononuclears and a few polymorphonuclear leucocytes Hemorrhage from damaged vessels is a prominent feature The bacilli gain access to the allantoic fluid in which they proliferate and are in contact with the entoderm, but no invasion of these cells occurs In embryos surviving 5 to 6 days the membranal lesion consists almost exclusively of mesodermal granulomata The bacilli are not demonstrable in great numbers The factors responsible for this proliferative reaction on the part of the mesodermal elements are not understood at present

The involvement of the endothelial cells lining the blood vessels leads to dissemination of the infection to the embryo by way of the circulating blood Although the majority of the embryos die during the 96 to 120 hour period, presumably because of this blood stream invasion, the surviving embryos sacrificed at this time do not all show evidences of localization in the form of focal lesions associated with the presence of the bacilli In a significant number of embryos this does occur and in these instances the infection proceeds by the localization of the bacilli within cells of various organs and most prominently in the liver, kidneys, spleen, and myocardium Focal areas of necrosis begin to appear during the 48 hour to 72 hour stage in the liver and spleen In the liver many of the endothelial cells lining the sinusoids and the hepatic parenchymal cells in these areas are greatly distended by large numbers of bacilli within their cytoplasm In the spleen the sinusoidal endothelium and especially the large mononuclears are filled with bacilli, apparently initial to areas of focal necrosis The glomeruli of both meso- and metanephros stand out prominently because of the marked invasion of their endothelial cells by the bacilli In the myocardium the focal lesions originate with the invasion of capillary endothelium Proliferative and necrotic changes due to invasion of vascular endothelium are present in various other organs and tissues

In some of the embryos which survived 5 and 6 days following inoculation granu-

omatous foci are present within the spleen. There are areas of focal necrosis in the liver. Perivascular granulomata are present in the meninges. A proliferative ependymitis has also been observed. Microorganisms have not been demonstrated in these lesions either intra- or extracellularly. More observations and further detailed study will be required before the pathogenesis of these lesions can be elucidated.

Brucella abortus

The observations on the behavior of this strain of *Br. abortus* are limited to one passage in the chorio-allantois.

Embryos infected by this route do not survive longer than 96 to 120 hours. The gross characteristics of the membranous lesion are indistinguishable from those produced by infection with *Br. suis*.

Microscopically, relatively slight reaction is observed in the chorio-allantois until 48 to 72 hours following inoculation. Practically no destruction of ectodermal epithelium has taken place although many of these cells are filled with microorganisms. In comparison with *Br. suis*, the microorganisms of this strain are present to a greater extent within mononuclears and fibroblasts of the mesoderm. Invasion of vascular endothelium is marked but does not constitute as prominent a feature. There is less destruction of endothelium and a proliferative reaction with the formation of endothelial thrombi is encountered much more frequently. In the later stages of the infection there is marked perivascular proliferation of fibroblasts forming granulomatous nodules.

Spread of the infection from the chorio-allantois to the embryo occurs during the period from 48 to 72 hours. This invasion is predominantly intracellular and can be observed to have spread along the endothelium of the large vessels leading from the membrane to the visceral vascular channels. Marked involvement of the liver, kidneys, spleen and myocardium results. Microorganisms prominently fill the cytoplasm of numerous endothelial cells lining the sinusoids of the liver and the capillaries of the kidney glomeruli. Extension of the infection to the hepatic parenchymal cells predominates the generalized infection. Focal areas of necrosis develop in the liver as a result of the parasitization of the cellular elements. In the spleen the sinusoidal endothelium presents the primary focus of localization with resulting focal areas of necrosis and fibroblastic proliferation. The occasional lesions observed in the myocardium also appear to develop as the result of capillary thrombosis secondary to localization of microorganisms within endothelial cells.

Brucella melitensis

This strain has thus far been studied through only one passage in the chorio-allantois.

Embryos have not survived the infection longer than 90 to 120 hours after inoculation. The gross appearance of the lesion is characterized by swelling and focal hemorrhages in the inoculated area within 24 hours. Much more inflammatory reaction develops than with *Br. suis* and *Br. abortus* and the infected membrane rapidly becomes opaque and thickened.

eralization of the disease can ensue. The chronicity of these infectious diseases might be well accounted for by the capacity of the microorganisms for intracellular growth, an environment which is generally regarded as uninfluenced by immune substances and by most therapeutic agents. That *B. tularense* and different strains of *Brucella* show selective affinities for different types of cells may be of epidemiological and certainly of pathological significance.

P. pestis exhibits less selectivity in its environmental requirements and proliferates with great rapidity in the tissue and circulating fluids. The behavior of this microorganism in the embryo can readily be correlated with that described in the human disease (12). In respect to its relationship to the host cell it exhibits a large measure of independence. It is evident from the appearance of the microorganism which under certain circumstances is present within the cytoplasm of ectodermal epithelial cells that the intracellular environment is inimical rather than favorable to its growth. As such it behaves as an extracellular parasite and this character combined with its adaptation for rapid proliferation and spread in various environments of this type differentiates it sharply from *B. tularense* and the *Brucella*.

Although tularemia has often been designated as a "plague-like disease" there is at present no agreement regarding the systematic position of *Bacterium tularense*. Bergey (13) classifies it as a *Pasteurella tularensis* and this designation has been accepted by Holmes (14) and others. Serological, cultural, and other considerations have led Francis (15) as well as Zinsser and Bayne-Jones (16) to place it in a semi-independent position as *Bacterium tularense*. Topley and Wilson (17) state that it probably should be assigned to the *Brucella* group.

While the present observations are not considered to constitute a basis for classification they indicate that in respect to its relation to the host cell *B. tularense* exhibits a behavior like that of the *Brucella* rather than that of *P. pestis*. The capacity for intracellular parasitism by *P. pestis* has not been observed in either the natural or experimental hosts or in its insect vector and does not constitute a prominent aspect of its behavior in the membrane. This microorganism is much less selective in its environmental requirements *in vivo* and is cultured much more readily on artificial media than are the *Brucella* and *B. tularense*.

Observations of this nature are primarily lessons in parasitism. They more strictly involve problems of host-parasite relationship which are pertinent at least during the early stages of the infectious process. Comparative studies of this type in the chick embryo in which the early stages of infection are readily accessible should bring out differences in behavior which may explain variations which occur in the natural disease produced by different strains of those microorganisms in man and animals. Thus from more extensive surveys it is to be hoped that a concept may be derived which would be more germane to the general problem of pathogenesis than has heretofore been possible.

SUMMARY

1 Comparison of the infections of chick embryos by the chorio allantoic route indicates that *Bacterium tularense* and *Brucella suis*, *abortus*, and *melitensis* exhibit varying degrees of facultative intracellular parasitism. *Pasteurella pestis* is adapted to rapid proliferation and spread in the intercellular fluids.

2 In the early stages of infection *Bacterium tularense* has a marked affinity for growth within ectodermal epithelial cells. *Brucella suis* and *Brucella abortus* differ in their selectivity for cells of mesodermal derivation and especially in their effect on vascular endothelium. The strain of *Brucella melitensis* studied is limited in its intracellular growth to ectodermal epithelium.

3 Many of the features characteristic of these infections in the natural hosts are reproduced in the chick embryo and its membranes.

4 The possible implications regarding the differences in behavior of these microorganisms in relation to the problem of infection and pathogenesis of these diseases are discussed.

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EXPLANATION OF PLATES

PLATE 10

FIG 1 Chorio-allantois 24 hour infection with *B tularensis* The infection at this stage is focal in character and limited to the ectoderm. A slight amount of inflammatory reaction and a few hemorrhages are present in the mesoderm. Wright's stain $\times 120$

FIG 2 Chorio-allantois 48 hour infection with *B tularensis* The infection has spread to the entire ectodermal layer of which all the cells are parasitized. There is necrosis of the underlying mesoderm. Most of the capillaries and small blood vessels are thrombosed. Wright's stain $\times 250$

FIGS 3 and 4 Ectodermal epithelial cells completely filled and distended with *B tularensis*. Wright's stain $\times 2000$

FIG 5 Chorio-allantois 96 hour infection with *Br suis* There is relatively slight injury to the ectodermal epithelium but the underlying capillary bed is sharply outlined by the presence of the bacilli within the endothelial lining cells. Wright's stain $\times 300$

FIG 6 Chorio-allantois 96 hour infection with *Br suis* Above are several parasitized ectodermal epithelial cells while directly beneath are endothelial cells lining the capillary plexus, which are filled with the bacilli. Wright's stain $\times 2000$

FIG 7 Chorio-allantois 72 hour infection with *Br suis* A large mesodermal vein with necrosis and proliferation of the endothelial lining. Wright's stain $\times 500$

FIG 8 Part of the endothelial lining of vein in Fig 7 showing the cells distended by the bacilli in their cytoplasm. Wright's stain $\times 2000$



PLATE 11

FIG 9 Chorio allantois 72 hour infection with *Br abortus* An artery showing the papillary proliferation of the endothelium There is less destruction of endothelium than in Fig 7 Hematoxylin and eosin stain $\times 120$

FIG 10 Proliferative reaction of endothelial lining almost occluding a small vein in chorio allantois following infection with *Br abortus*, 72 hours Hematoxylin and eosin stain $\times 550$

FIG 11 Liver from embryo infected by way of the chorio allantois with *Br abortus* Many of the endothelial cells lining the sinuses and the liver parenchymal cells are conspicuous because they are densely packed with bacilli Wright's stain $\times 225$

FIG 12 An endothelial cell lining a liver sinus filled with *Br abortus* Wright's stain $\times 2000$

FIG 13 A liver parenchymal cell filled with *Br abortus* Wright's stain $\times 2000$



THE ROENTGEN RADIATION OF PAPILLOMA VIRUS (SHOPE)

II THE EFFECT OF X RAYS UPON PAPILLOMA VIRUS IN VITRO*

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A benign, infectious, cutaneous tumor of rabbits, the Shope papilloma, is caused by a virus that upon inoculation in either domestic or cottontail rabbits induces papillomas with great regularity (1). These tumors are not infrequently followed by cancers (2-6). On attempted recovery of the virus from these two varieties of rabbits, however, a marked difference in host reactivity becomes apparent, for the virus, which is readily recoverable from cottontail rabbits, is not recoverable, or but rarely recoverable, from domestic rabbits (1, 7-9). This finding does not prove, of course, that the virus is absent from the papillomas of domestic rabbits. On the contrary, some immunological evidence for its presence in such papillomas and in the cancers derived from them has been presented (10, 11).

If a virus were not present in the cells of papillomas on domestic rabbits, such papillomas might be expected to react to Roentgen radiation as non infectious tumors do. This, indeed, has been shown to be the case (12). On the other hand, if a virus were contained in the tumor cells, the outcome following irradiation might be expected to be quite different. Obviously, therefore, it is important to learn the effect of Roentgen radiation on the virus itself. Data bearing on these points have been briefly recorded by Lacassagne (13) and ourselves (14, 15). The work from our laboratory that dealt with the effects of Roentgen radiation on papillomas *in vivo* has been amplified by further experimentation and presented in full (12). It was concluded that the curative effect of x rays on domestic rabbit papillomas (Shope) results from the direct action of the Roentgen radiation on the rabbit's cells rather than on the virus.

The studies described in the present paper deal with the effect of Roentgen radiation *in vitro* on cell free suspensions of papilloma virus derived from cottontail rabbits. The experiments were designed to determine the dosage necessary to render papilloma virus non infectious for both cottontail and domes-

* The present investigation was aided in part by grants from The Jane Coffin Childs Memorial Fund for Medical Research and the International Cancer Research Foundation.

tic rabbits They were planned, also, to detect any manifest alterations, if such occurred, in the characteristics of papillomas that were initiated by irradiated virus

Materials and Methods

Virus—The papilloma virus (Shope) was obtained from papillomas that had been incurred under natural conditions in Kansas by cottontail rabbits (genus, *Sylvilagus*) Immediately after the removal of papillomatous tissue from the rabbits, it was thoroughly triturated with Locke's solution and alundum¹ to yield a 10 per cent solution This suspension was centrifuged horizontally at 1500 R P M for 30 minutes and the supernatant fluid withdrawn by means of a pipette Care was exercised not to disturb the sediment and to leave behind the debris floating on the supernatant fluid The collected fluid was then subjected to three successive centrifugations at 14,000 R P M (relative centrifugal force at tip of tube approximately 25,000 \times gravity) in the 51°-angle head of the Multispeed Attachment for the International centrifuge, the sedimented material being discarded after each run The final supernatant fluid, either without further treatment or after passage through a Berkefeld V filter, was used as the suspension of virus to be irradiated Since filtration often results in a 10-fold decrease in the titer, the suspension was not filtered in the present work, except in the first two experiments

Method of Radiation—The suspension of virus to be irradiated was transferred to a sterile Petri dish, 4 cm in diameter and 8 mm in depth To prevent contamination and evaporation, the dish was covered by a sterile piece of emulsion-free dental x-ray film This film was used because it has essentially no filtering effect upon the x-rays The dish was placed on a small, lead-covered table that rotated slowly under a shielded, water-cooled radiographic tube especially adapted to this purpose During irradiation, at intervals computed to yield the dosages desired, the suspension of virus was mixed thoroughly and 0.5 ml samples were removed for testing The irradiation of any given sample was continuous The use of the loosely covered Petri dish made it unnecessary to shield the suspension from the radiant energy of the filament, for the temperature of the suspension was never found to exceed the temperature of the laboratory by more than 3°C throughout the entire period of irradiation

The Roentgen rays were generated by a current of 40 milliamperes at 90 kilovolts The radiation, which was unfiltered, was administered at a distance of 6 cm from the center of the target to the center of the Petri dish, a point 4 mm from the bottom of the dish Although entirely accurate measurements of dosage at this short distance and high output proved to be difficult, a Victoreen r-meter, calibrated for 90 kilovolts, was used to determine the dosage The ionization chamber (1 cm in diameter) of the calibrated Victoreen instrument was placed horizontally in a Petri dish This Petri dish was identical with the one used for the suspension of virus, except that one

¹ Alundum, an electrically fused crystalline alumina prepared by the Norton Company, Worcester, Massachusetts, was used because of its excellent "cutting" qualities

side was chipped out to permit the chamber to rest in it. To reproduce the conditions of the actual test still more closely, a cover of emulsion free dental x ray film was placed over the container. Thus the conditions for the measurement of dosage were the same as for the actual tests except for a possible scattering of the x rays by the suspension of virus. To measure the output of radiation the radiographic tube was centered above the ionization chamber at distances of exactly 96 cm, 24 cm, 12 cm, and 6 cm respectively. Short exposures were controlled by a radiographic interval timer. The output of radiation, including back scattering was measured for each experiment. The readings showed little or no deviation from 10,400 r units per minute at a distance of 6 cm. Although the inverse square law may not hold at very close distances, the actual readings that were obtained at a distance of 6 cm corresponded to the theoretical readings calculated by means of the inverse square law.

In an effort to render successive doses of x rays as comparable as possible, all geometric factors were kept approximately constant. The only variables were the level of the suspension of virus in the Petri dish and the time of exposure which was measured in seconds by both a timer and a stop watch. The error in the measurement of dosage was approximately ± 5 per cent. The difference in dosage that was received at the top of the suspension and at the bottom, a difference of 8 mm, was calculated by the inverse-square law to be 7.5 per cent. Since the physical limitations of size make such changes in dosage unavoidable, however the doses as given are comparable for the factors as specified.

Animals—130 adult rabbits were used in the present study. 16 cottontail rabbits (*Sylvilagus floridanus alacer* Bangs) from Kansas provided the virus for irradiation, 39 cottontail rabbits (*Sylvilagus floridanus mearnsi* Allen²) from New York and 75 domestic rabbits (genus *Oryctolagus*) served as host animals for testing the infectivity of the irradiated samples of virus. The domestic rabbits were employed to determine the titer of the suspensions before irradiation and to measure any loss in titer that might follow irradiation. The cottontail rabbits made it possible to determine the 'viability' of the virus in each irradiated sample since papilloma virus is readily recoverable from this host.

Technique for Measuring the Viability and Titer of the Irradiated Virus—After exposure to each of the specified doses of x rays, a 0.5 ml sample of the suspension of virus was withdrawn and diluted with Locke's solution to yield a series of decimal dilutions through 10^6 . Each dilution was used for duplicate triplicate, or quadruplicate inoculations made by rubbing 0.1 ml on a previously prepared lightly scarified cutaneous site (approximately 2 cm in diameter) on the ventral surface of the body. The size of the rabbit determined the number of sites. Domestic rabbits readily accommodated 16 rectangular areas, 4 cm square cottontails from 8 to 12 such areas. The rabbits were observed at intervals of from 2 to 7 days for a period of 60 days a procedure that permitted a satisfactory assessment for 'viability' and titer of each sample of irradiated virus.

² It is a pleasure to thank Mr George G Goodwin of The American Museum of Natural History New York City, for making the specific allocation of the cottontail rabbits used.

indicated in Table III. The virus in each sample was tested as in Experiment 2 by the inoculation of 4 animals, 2 cottontail rabbits and 2 domestic rabbits.

The results of the third experiment are set forth in Table III. Dosages of less than 2 million r had no observable effect, 2 million r probably had elicited some effect, as shown by some prolongation of the incubation period and possibly by a 100-fold loss in titer, and 3 million r, or more, resulted in from a 10- to 100,000-fold loss in titer and in a change from an incubation period of 12-32 days to 15-34 days. The effect of 4 million r was more pronounced. Finally, 5 million r inactivated most of the virus, for only a single cottontail of the 4

TABLE III

The Results of Single Massive Doses of X-Rays Ranging from 500,000 to 7 Million r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test viability ¹ and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
500	1	2	12-23	10^4-10^5	0 -
1000	2	2	15-23	10^4-10^5	0
2000	2	2	15-23	10^3	0-100 fold
3000	2	2	15-26	10^4-10^2	10-10,000 fold
4000	2	2	26-34	10^1	100-10,000 fold
5000	2 (2)*	2 (1)	26	10^1	100-10,000 fold
6500	2 (2)	2 (2)	—	—	1000-100,000-fold
7302	2 (2)	2 (2)	—	—	1000-100,000-fold
Control		2	15	10^3-10^5	

* Numbers in parentheses signify the number of rabbits that gave no reaction following the inoculation of irradiated virus.

cottontail rabbits that received virus irradiated with this dosage reacted to the undiluted material (at only 2 of 4 sites). Doses of approximately 6 and 7 million r were uniformly effective in inactivating the virus. Virus was regularly recovered from the papillomas induced on cottontail rabbits.

In evaluating the effect of irradiation in the third experiment, it should be noted that one of the domestic rabbits employed to titrate the suspension of virus for irradiation responded to the virus in a normal manner, while the other was relatively refractory to infection, as indicated by the low titer and by the fact that the papillomas regressed completely soon after their appearance. Such a regression rarely occurs in the domestic rabbit within 6 weeks.³ An

³ The decreased reactivity of one of these 2 domestic rabbits to infection by the virus may possibly be attributed to an increased tissue resistance, for the neutralizing

initial titer of 10^5 , therefore, would probably represent the infectivity of the material more closely than 10^3

A fourth experiment was undertaken to determine the reproducibility of the results obtained in Experiment 3, using a different preparation of virus

Experiment 4—A suspension of the virus was irradiated continuously until a dosage of 14,325,000 had been reached. During the period of irradiation, 4 samples representing dosages of 1 500,000 r, 5,025 000 r, 13,875,000 r, and 14 325,000 r were withdrawn and tested, as indicated in Table IV. The virus in each sample was tested as in Experiment 2 by the inoculation of 4 animals, 2 cottontail rabbits and 2 domestic rabbits. Three of the 4 cottontail rabbits used to test the samples of virus irradiated

TABLE IV

The Results of Single Massive Doses of X Rays Ranging from 1.5 to 14 Million r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test viability and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
1,500	0	2	14	10^2 - 10^6	0-10 000-fold
5,025	1	2 (1)*	30-41	10^1 - 10^2	10 000-1 million fold
13 875	2 (1)	2 (2)	30	10^1	100 000-1 million fold
14 325	2 (2)	2 (2)	—	0	
Control	0	3	10-13	10^5 - 10^6	

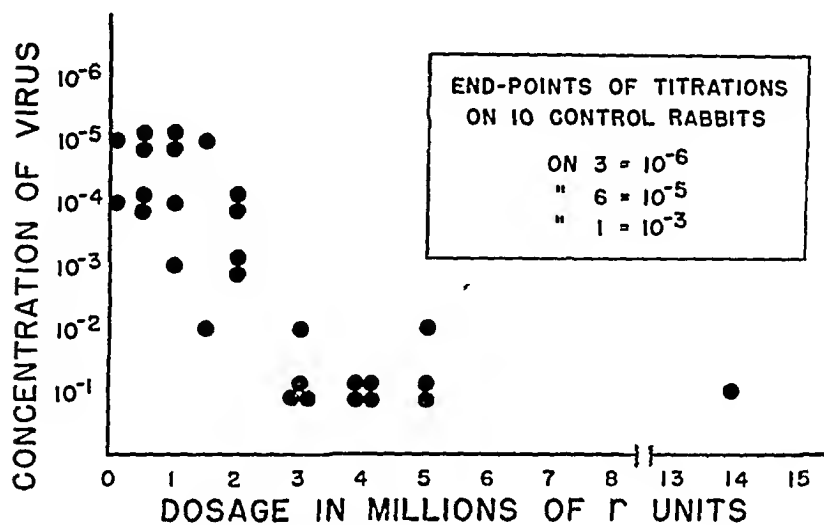
* Numbers in parentheses signify the number of rabbits that gave no reaction following the inoculation of irradiated virus

with 1 500,000 r and 5,025,000 r died from extraneous causes before papillomas developed.

The results of the fourth experiment are summarized in Table IV. It may be seen that the results obtained in Experiment 3 were confirmed and extended. A dose of a million and a half r was without obvious effect. A dose of 5 million r, on the other hand, resulted in a significant loss of infectivity (from 10,000- to 1,000,000-fold) and in a prolongation of the incubation period (from 10-13 days to 30-41 days). Moreover, papillomas were induced on only a single rabbit of the 4 that received material irradiated with the enormous dosage of almost 14 million r. On this single animal, furthermore, papillomas appeared at but 2 of the 4 sites inoculated, and then only after a prolonged incubation period of 30

capacity of sera withdrawn from the 2 animals before inoculation was identical as was the capacity of similar samples withdrawn 60 days after injection. Nevertheless, the papillomas on one rabbit had regressed completely within 40 days whereas those on the other rabbit grew progressively as usual.

days (Because this positive finding concerns a single animal only, one must not overlook the possibility that it may have been exceptional) With the next larger dose, over 14 million r, no reaction in any of the injected rabbits was



SUMMARY

NUMBER OF RABBITS DEVELOPING PAPILLOMAS

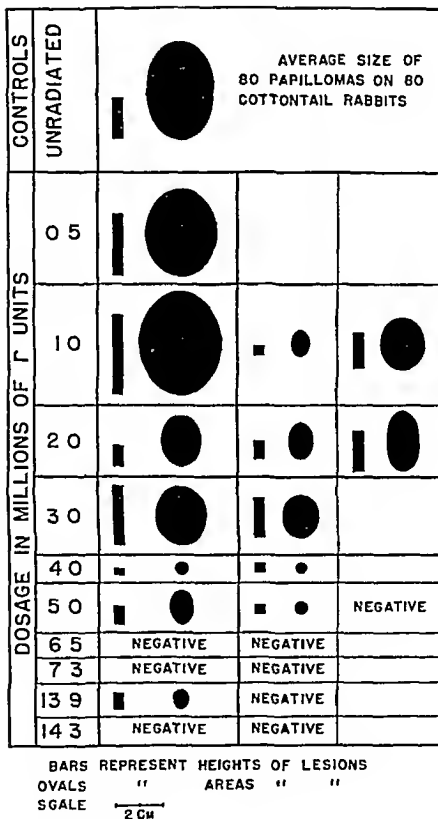
$\frac{2}{2}$	$\frac{4}{4}$	$\frac{4}{4}$	$\frac{2}{2}$	$\frac{4}{4}$	$\frac{4}{4}$	$\frac{4}{4}$	$\frac{3}{7}$	$\frac{0}{4}$	$\frac{0}{4}$			$\frac{1}{4}$	$\frac{0}{4}$
0	1	2	3	4	5	6	7	8	13	14	15		

DOSAGE IN MILLIONS OF r UNITS

TEXT-FIG 1 The effect of Roentgen radiation on the infectivity of papilloma virus in cell-free suspensions. Each dot shows the highest titer attained on an individual positive rabbit. The concentration of the virus is plotted against the dosage of x-rays, a concentration of 10^{-1} equalling a 10 per cent suspension of tumor tissue. In the summary at the bottom of the figure, the lots of animals inoculated with each sample of irradiated virus are recorded as the denominators of the fractions, those developing papillomas as the numerators.

noted. Virus was regularly recovered from papillomas induced in cottontail rabbits.

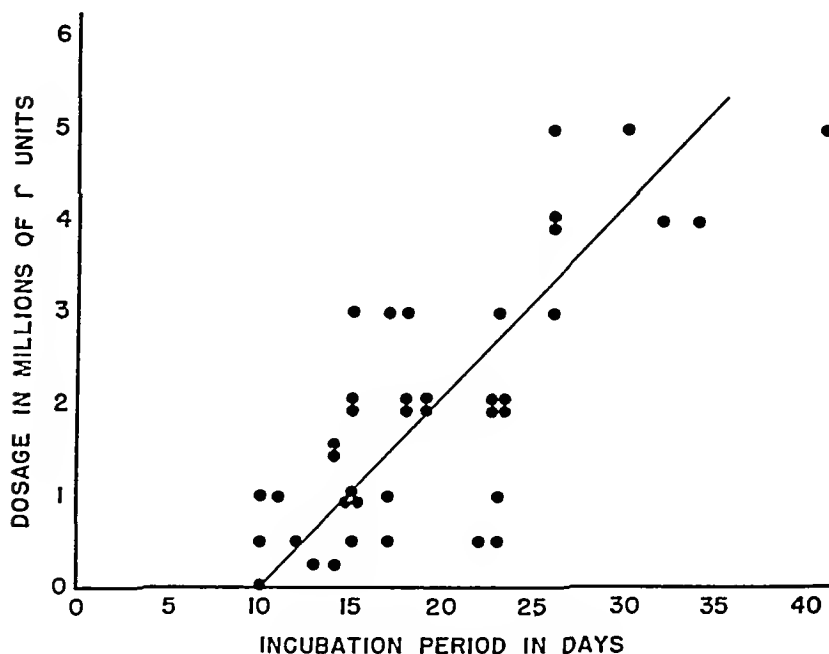
When the four experiments were considered collectively, it was evident that large doses of Roentgen radiation result in a lowered titer, a prolongation of the incubation period, and a decrease in the size attained by the papillomas. These effects are readily apparent when the results that follow irradiation with graded doses of from 100,000 to 14 million r are depicted graphically. In Text-fig 1



TEXT FIG. 2 The sizes attained by papillomas on cottontail rabbits inoculated with irradiated papilloma virus. Each rectangular area of the figure represents an individual rabbit (except in the case of the controls). The effect of the x rays in decreasing the size of the tumors is apparent.

the loss in the infective capacity of the virus that occurred when the dosage of radiation was increased is graphically shown. (The results of Experiment 1,

the preliminary experiment, are not included) It may be noted that a demonstrable effect on the virus was not elicited until the dosage had reached 1 million r With increasing dosage, the reduction in the infective titer was progressively greater In Text-fig 2 the progressive decrease in the size of the tumors that resulted from the inoculation of the irradiated virus (in 10 per cent suspension) is shown The effect on the size of the papillomas following successively greater dosages of x-rays is obvious Other variations from norma



TEXT-FIG 3 The effect of x-rays in prolonging the period of time between the inoculation of rabbits with irradiated papilloma virus and the appearance of the tumors The dosage of x-rays is plotted against the incubation period

accompanied the progressive decrease in the size of the tumors Thus, the incubation period was prolonged from 10 to 41 days, as shown in Text-fig 3 (In this text-figure, as in the others, the graphs necessarily represent approximations, for the variation that characterizes the host-virus relationship renders a more precise representation impossible) Although it is difficult to measure it is our impression that the "viability" of the papilloma cells may have been affected when the virus used for inoculation had received large doses of x-rays, for the resultant tumors frequently were dry and regressed early Such tumors, nevertheless, when present on cottontail rabbits, regularly yielded virus

DISCUSSION

In the present experiments the results have clearly shown that cell free suspensions of papilloma virus (Shope) require for their complete inactivation amounts of Roentgen irradiation much greater than those needed to inactivate other infectious agents. These amounts, millions of r units, are several thousand times greater than those required to bring about the permanent regression of tumors induced by the virus in domestic rabbits (12), and they are many times greater than those needed for the inactivation of certain other viruses, bacteria, and a yeast (14). The reason for this greater resistance to x-rays is not known.

Friedewald and Anderson (16) have recently reported studies that appear to be similar to ours. They found that papilloma virus, when contained in Berkefeld filtrates, is inactivated by from 2 to 4 million r, whereas preparations of virus partially purified by repeated differential centrifugations require only from 400,000 to 800,000 r for inactivation. They attribute this difference to the greater concentration of extraneous material and virus in Berkefeld filtrates. Quite possibly the same explanation might account, at least in part, for the larger doses that were required in our investigations. It seems not unlikely, however, that the differences in the results obtained by Friedewald and Anderson and by us were caused in part by differences in the methods of irradiation and in the animal hosts used for testing the irradiated samples of virus. We used continuous irradiation and employed only a single x ray tube. Each irradiated sample of virus was tested for "viability" and titer using domestic and cottontail rabbits. It should be remembered that the cottontail rabbits used for testing the irradiated samples were of the Eastern subspecies (*Sylvilagus floridanus mearnsi* Allen), a host that is uniformly highly susceptible to the virus. As a rule, it reacts more profusely and to a higher titer with a given sample of virus than do either domestic rabbits or Western cottontail rabbits (*Sylvilagus floridanus alacer* Bangs). Papillomas induced on the Eastern cottontail rabbit, furthermore, readily yield virus for further study. It should be noted, finally, that it was on this host that papillomas were produced with the samples of virus irradiated with the greater dosages.

It is apparent from the present work that the Roentgen irradiation of materials containing papilloma virus provides a method whereby bacterial invaders can be eliminated from papillomatous material. The bacteria will be destroyed by 2 million r without observably injuring the papilloma virus. The same procedure may be useful in limiting extraneous viruses, for several are known to be inactivated by this amount of irradiation (14, 15). Using these large doses of x rays, the treatment of the cancers that often follow the papillomas are providing us currently with a further approach to the study of the rôle of papilloma virus in the papilloma to-carcinoma sequence.

CONCLUSIONS

Cell-free suspensions of papilloma virus (Shope) required for their inactivation *in vitro* amounts of Roentgen irradiation that are much greater than those needed to inactivate other infectious agents previously described. These amounts, millions of r units, are several thousand times greater than those required to eradicate permanently papillomas induced by the virus in domestic rabbits. Large doses of Roentgen radiation reduce the titer of papilloma virus, lengthen the period of time between inoculation and the appearance of papillomas, and decrease the size attained by the papillomas.

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RENAL HYPERTENSION PRODUCED BY AN AMINO ACID*

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PLATE 13

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Holtz (1, 2) and Bing (3, 4) showed that the anaerobic incubation of extracts of the renal cortex with amino acids converted these substances into their corresponding amines. The presence of the amines was demonstrated by their effect on the blood pressure of the cat or by their chemical isolation (1, 2). Holtz demonstrated that the amines were produced from amino acids by the action of decarboxylating enzymes contained in the renal cortex. The reaction occurred only under anaerobic conditions. When the incubation was carried out in the presence of oxygen the amino acids were converted, probably by deamination, into substances differing pharmacologically and chemically from amines. Holtz *et al* (5) and Bing and Zucker (4) found that the amino acid decarboxylases were specific for certain amino acids and varied with the animal species.

Bing (3) demonstrated that the isolated ischemic kidney of the cat perfused *in vitro* with blood containing *l*-dopa (*l*-dihydroxyphenylalanine) transformed this amino acid into hydroxytyramine by decarboxylation. Since this amine in contrast to dopa, is a pressor substance it was possible to demonstrate its presence by the injection of the perfusion fluid into a cat. The amount of amine produced by the kidney varied inversely as the rate of blood flow. Only negligible amounts of pressor substance were found in the perfusate when the renal blood flow was normal.

It is generally believed that in experimental hypertension the kidney elaborates a substance causing arteriolar constriction. The conditions under which the pressor substance is formed are believed to be renal ischemia (6) or a reduction in the renal pulse pressure (7). The fact that the ischemic kidney converts dopa, which has no effect on the blood pressure, into a pressor substance, presumably hydroxytyramine, suggested that renal decarboxylation might play some part in the etiology of hypertension. The purpose of the present investigation was to ascertain whether or not hypertension could be produced in animals by the intrarenal conversion of dopa into a pressor substance.

Methods

In all experiments cats anesthetized with nembutal (39 mg per kilo body weight, intraperitoneally) were used. The kidneys and their pedicles were exposed retro-

* Part of this paper was presented before the American Physiological Society, Chicago 1941.

peritoneally In some experiments the collateral circulation through the capsule and ureter was left intact, in others it was destroyed by freeing the kidney from the peritoneum and fat, tying and cutting the ureter, and dissecting the pedicle No attempt was made to leave the renal nerves intact Complete renal ischemia was achieved by the application of serrefines to both renal pedicles for a period of from 2 to 4 hours Dopa was injected into the left kidney, the right kidney serving as a control Partial ischemia was created by placing a Goldblatt clamp (8) on the left renal artery The right pedicle was clamped throughout the experiments and dopa was injected into the left kidney From 10 to 50 mg of *l*-dopa (*l*-dihydroxyphenylalanine)¹ dissolved in 3 to 5 cc of Ringer's solution were used, in the majority of experiments 10 mg in 3 cc were injected The solutions were freshly prepared

Two alternative methods of injecting the dopa solution were employed In some instances the fluid was injected by syringe through the renal capsule into the kidney tissue, in others into the renal blood supply through the aorta In the latter method, the renal vein and the aorta above and below the origin of the renal artery were clamped with serrefines The dopa solution was injected into the left renal artery through a 26 gauge needle inserted into the lumen of the aorta below the renal artery After the injection, a third clamp was placed on the aorta proximal to the site of the injection and the needle was withdrawn The serrefine on the aorta above the renal artery was removed to wash the dopa solution into the kidney and 20 seconds later the renal artery was clamped Immediately afterwards the remaining clamps on the aorta were removed The aorta was compressed for 5 minutes with a gauze tampon to prevent hemorrhage from the point of injection The circulation through that vessel was then reestablished A slightly different procedure which will be described below was employed in the experiments on the partially ischemic kidney

The release of the pressor substance formed from dopa was detected by its effect on the blood pressure, measured from the carotid artery by a mercury manometer

EXPERIMENTAL

Experiments on the Completely Ischemic Kidney

Thirteen experiments were performed to investigate whether the completely ischemic kidney is able to convert dopa into a pressor substance

Dopa was either injected into the substance of the left kidney or into the blood supply of that organ through the aorta 2 to 4 hours after the blood supply had been interrupted, measurement of the blood pressure was started The clamp was removed from the pedicle of the uninjected kidney, restoring its circulation 10 to 15 minutes later the circulation to the injected organ was similarly reestablished

In every instance the removal of the clamp from the pedicle of the kidney containing the dopa solution was followed by a rise in blood pressure (Fig 1) The peak of the pressure rise was reached within 2 minutes after the restoration of the circulation The elevation varied from 15 to 115 mm Hg (Table I) The wide variation was probably due to the loss of small amounts of dopa solu-

¹ Hoffmann-La Roche, Inc.

TABLE I
Experiments on the Completely Ischemic Kidney

Date	Control kidney			Injected kidney			
	Collateral circulation	Duration of ischemia	Rise in blood pressure	Dopa injected	Injected into	Duration of ischemia	Rise in blood pressure
12/ 6/40		5 hrs 6 min	mm Hg 35	mg 50	Aorta and tissue	4 hrs, 30 min	mm Hg 40
12/ 7/40		2 hrs 50 min	5*	50	Aorta and tissue	2 hrs 45 min	85
1/25/41	Present	3 hrs 8 min	10 mm fall then 100 mm rise†	10	Aorta	2 hrs., 55 min	10 mm fall then 100 mm rise
1/27/41	Absent	4 hrs	45	10	Aorta	4 hrs	18
2/ 4/41	Absent	4 hrs 17 min	110				
2/ 6/41	Present	2 hrs 42 min	30 mm fall	10	Tissue	2 hrs, 45 min	15 mm fall then 30mm rise
2/ 6/41	Present	2 hrs. 30 min	15*	10	Tissue	50 min	45
2/ 7/41	Present	3 hrs, 20 min	0	10	Tissue	3 hrs. 8 min	20 mm fall then 80mm. rise
2/10/41	Absent	3 hrs	5*	10	Tissue	2 hrs 49 min	115
2/11/41	Absent	3 hrs 30 min	10*	10	Tissue	3 hrs. 13 min	95
2/11/41	Absent	3 hrs. 6 min	0	10	Tissue	2 hrs, 53 min	0‡
2/22/41	Absent	3 hrs 4 min	0	10	Tissue	1 hr 43 min	20 mm fall then 75mm. rise

* Rises occurred within 8 seconds probably due to increase in blood volume

† Dopa probably reached the kidney through its collateral circulation

‡ Circulation could not be reestablished since a clot had formed in the renal artery

tion during the injections or to differences in the ability of the kidneys to decarboxylate dopa

In eight experiments the reestablishment of the circulation to the uninjected

from dopa was identical with hydroxytyramine since (a) Holtz crystallized this amine after the anaerobic incubation of renal cortical extracts with dopa, (b) the kidney formed a pressor substance during 3 hours of ischemia only when dopa was present, (c) the pressor substance formed from this amino acid was heat-stable and ultrafilterable, and (d) its effect was potentiated by cocaine

Experiments on the Partially Ischemic Kidney

A series of 40 experiments was performed to investigate whether the partially ischemic kidney could transform dopa into the pressor substance. In 29 experiments the dopa solution was injected into the left kidney through the aorta, in 11 cases, into the renal parenchyma. The contralateral kidney was clamped

TABLE III
The Effect of Cocaine on the Pressor Substance

Date	Renal pedicle unclamped before the injection of cocaine		Renal pedicle unclamped after the injection of cocaine	
	Duration of ischemia	Rise in blood pressure	Duration of ischemia	Rise in blood pressure
		<i>mm Hg</i>		<i>mm Hg</i>
1/10/41	2 hrs, 28 min	55	2 hrs, 42 min	65
1/10/41	2 hrs, 22 min	20	2 hrs, 17 min	75
1/20/41	2 hrs., 12 min	25	2 hrs, 8 min	20 mm fall then 90 mm rise
1/21/41	3 hrs, 11 min	10	2 hrs, 58 min	40
4/ 1/41	3 hrs, 30 min	30	3 hrs, 30 min	40

in every instance to avoid the destruction of hydroxytyramine which occurs in kidneys with normal blood flow (3)

In 29 experiments the right renal pedicle or the right renal artery alone was clamped with a serrefine. The left kidney and its pedicle were exposed and the abdominal aorta was dissected for approximately 1 cm on either side of the origin of the renal artery. A Goldblatt clamp was placed on the renal artery, but was not tightened until after the injection of the dopa solution. Serrefines were placed on the renal vein and on the aorta above and below the origin of the renal artery. 10 mg of dopa dissolved in 3 cc of Ringer's solution were injected through the aorta as described above. The serrefines on the aorta were removed and the aorta was compressed with a gauze tampon until bleeding from the point of injection had ceased. The Goldblatt clamp was then adjusted to obtain partial renal blood flow. Finally the serrefine on the renal vein was removed. Compression of the aortic wound made complete renal ischemia lasting from 2 to 13 minutes unavoidable.

In some instances one animal was used for a series of injections. Control experiments in which Ringer's solution instead of dopa was injected were performed in a similar fashion.

In 15 instances the injection of dopa into the renal artery was followed by a rise in blood pressure averaging 100 mm Hg (Table IV). In 11 of these experiments the curves obtained were steep and the peaks were reached 40 seconds after the partial restoration of the renal blood flow. In one instance in which 4 minutes elapsed between the injection of the dopa solution and the restoration

TABLE IV
*Experiments on the Partially Ischemic Kidney**

Date	Rise in blood pressure	Duration of complete ischemia	Remarks
	mm Hg	min	
12/21/40	100	11	Initial rise of 40 mm Hg. Further rise of 60 mm when Goldblatt clamp was opened.
12/26/40	120	2	Blood pressure rose slowly 20 mm. Further opening of the Goldblatt clamp was followed by a rapid rise of 100 mm Hg.
1/15/41	130	4	
1/16/41	110	6	
2/14/41	150	13	Initial rise of 60 mm Hg. Further rise of 90 mm when Goldblatt clamp was opened.
2/15/41	120	6	
2/24/41	70	12	
2/25/41	120	4	
2/26/41	90	1 5	Blood pressure rose slowly 25 mm. Further opening of the Goldblatt clamp was followed by a rapid rise of 90 mm Hg.
2/28/41	80	8	Blood pressure rose slowly.
3/ 1/41	100	9	
3/ 3/41	100	10	
3/ 3/41	100	3	Initial rise of 40 mm. Further rise of 60 mm when Goldblatt clamp was opened.
3/ 4/41	75	2	Blood pressure rose slowly 30 mm. Closing clamp caused fall to control levels. Fig 4 shows subsequent changes in blood pressure.
3/11/41	70	9	

* In these experiments 10 mg dopa in 3 cc Ringer's solution were injected through the aorta. For negative results see text.

of the renal circulation the rise amounted to 120 mm Hg. In four experiments more gradual rises were observed (Figs 2 to 4).

The pressor substance was not formed from dopa in the partially ischemic kidneys of four cats. In three of these animals the kidneys were possibly deficient in dopa decarboxylase, in the fourth the renal artery was not found to be patent at the end of the experiment. In other instances in which an injection of dopa was not followed by a pressor response, a previous or subsequent

injection gave a positive result. It is probable that the dopa solution had not reached the kidney in these experiments.

In four experiments in which Ringer's instead of dopa solution was injected, no rises in blood pressure were observed. This indicated that the reduction of renal blood flow alone could not produce acute renal hypertension, a result which is in agreement with that obtained by Schroeder (12).

In eleven experiments the amino acid was injected into the parenchyma of the partially ischemic kidney. This method reduced the period of complete renal ischemia which existed between the injection of dopa and the reestablishment of the renal circulation to less than 30 seconds.

A Goldblatt clamp was placed on the left renal artery and tightened immediately. A serrefine was placed on the left renal vein and 10 mg of dopa dissolved in 3 cc of Ringer's solution were injected into the renal tissue. The Goldblatt clamp was slightly opened and the serrefine removed from the vein.

The injection of dopa was followed in four experiments by a slow rise in the blood pressure, amounting to 35 mm Hg in three cases and to 65 mm in the fourth. The rises extended over a period of 4, 16, 11, and 35 minutes respectively. When leakage of the injected solution occurred following the withdrawal of the needle, as it did in six instances, the injection of dopa had no effect on the blood pressure. The degree of ischemia was of importance for the formation of the pressor substance. This was indicated in one experiment in which the blood pressure rose only 25 mm Hg after the injection of dopa. Following slight tightening of the clamp, however, an additional elevation of 40 mm Hg occurred, lasting 35 minutes. Complete closing of the clamp resulted in a fall in blood pressure to its control level. In a second experiment a rise from 110 to 145 mm Hg, lasting for 37 minutes, occurred during partial renal ischemia. In this case, as in the preceding one, occlusion of the renal pedicle caused a return of the blood pressure to its control level. These experiments indicated that dopa had been transformed into the pressor substance during partial renal ischemia.

Experiments on Kidneys with Normal Blood Flow

Nine experiments were performed to ascertain whether the kidney with normal blood flow was able to form the pressor substance from dopa.

In four experiments 10 mg of dopa dissolved in 3 cc of Ringer's solution were injected into the renal tissue, without interruption or reduction of the renal circulation. In five instances the solution was injected through the aorta in the following manner. After placing a hemostat on the aorta below the renal artery, the dopa solution was injected. A second hemostat was placed above the point of injection, slightly below the renal artery, for the remainder of the experiment, thus entirely abolishing the period of complete ischemia. Handling and dissection of the kidney and its pedicle were avoided as far as possible.

In three instances no change in blood pressure was noticeable after the injection of the dopa solution into the renal blood supply. In two experiments, however, the blood pressure rose 25 mm Hg, the rise extending over a period of 6 minutes. Since in these cases the kidneys had been slightly moved, the formation of the pressor substance, if any occurred, may have been the result of renal ischemia following traumatic vasoconstriction. The injection of dopa into the kidney substance had no effect on the blood pressure in three out of four cases. In the fourth instance a rise of 15 mm Hg occurred within 2 minutes after the injection. The animal on which this experiment was performed, however, showed spontaneous fluctuations in blood pressure. It was evident from these experiments that no pressor substance is formed from dopa when the renal circulation is completely unimpaired. The injection of the amino acid following slight trauma of the kidney, however, appears to result in the production of the pressor substance.

DISCUSSION

Experiments in which dopa (*L*-dihydroxyphenylalanine) is injected into completely ischemic kidneys of cats demonstrate that this amino acid is converted into a strong pressor substance. After 2 to 4 hours of ischemia, the rises in blood pressure observed following the reestablishment of the circulation to the injected kidney vary from 15 to 115 mm Hg. The removal of the clamp on the pedicle of the uninjected kidney after 2 to 3 hours of ischemia does not cause a rise in the blood pressure either in the presence or absence of the collateral renal circulation. When the blood supply is restored following 4 to 5 hours interruption, however, pressor effects amounting to 110 mm Hg are observed. The formation of a pressor substance in the uninjected, completely ischemic kidney has been reported by several workers. Rises in blood pressure have been observed in dogs after $\frac{1}{2}$ to 24 hours of renal ischemia (13-16), in the cat after 4 to 6 hours (17). The effect of shorter periods of ischemia has not been previously reported in the cat. Since the interruption of the blood supply to the uninjected kidney for 2 to 3 hours does not lead to the formation of a pressor substance, the effects observed following the injection of dopa into the completely ischemic kidney of the cat must be caused by the transformation of this amino acid into a pressor substance.

The observation that this substance is heat-stable and dialyzable differentiates it from the protein like pressor substance, resembling renin, which is said to originate in the completely ischemic kidney of the cat (17). On the basis of the work of Holtz the assumption might be ventured that the pressor substance formed from dopa is the amine hydroxytyramine as it originates only in ischemic kidneys containing dopa and its effect is enhanced by cocaine.

Rises in blood pressure are similarly observed after the injection of dopa into kidneys made partially ischemic by the application of a Goldblatt clamp. It is conceivable that the pressor substance is formed during the period of com-

plete renal ischemia existing between the injection of the dopa solution and the reestablishment of the renal circulation rather than during the period of partial ischemia. Rises in blood pressure are observed, however, in experiments in which partial ischemia was caused by traumatic vasoconstriction provoked by handling of the organ. It is evident, therefore, that the partially ischemic kidney can convert the amino acid into the pressor substance, presumably hydroxytyramine. This conclusion is supported by the work of Bing (3) on the perfused organ. The importance of renal ischemia for the production of the pressor substance is demonstrated in experiments in which the injection of dopa into kidneys with normal blood flow failed to produce any change in the blood pressure.

The connection between Goldblatt hypertension (8) and the hypertension produced by the injection of dopa is as yet only hypothetical. It is possible, however, that the transformation of dopa into a pressor substance by decarboxylation represents the pattern of events taking place in the hypertensive kidney. According to this concept, hypertension would be caused by an interference with the normal enzymatic breakdown of amino acids and amines in the kidney. This organ contains specific amino acid decarboxylases (4) and an unspecific deaminase (18). Any decrease of the oxygen consumption of the kidney would inhibit deaminization, since oxygen is a necessary factor in this reaction (19). Decarboxylation, however, being an anaerobic process, would occur, leading to the formation of substances similar to hydroxytyramine in chemical constitution and pharmacological action. Rodbard and Katz (20) found that the normal kidney destroys the pressor substances present in experimental hypertension by metabolic processes not connected with its excretory function. It is possible that this destruction occurs by deaminization. The observation of Mason, Robinson, and Blalock (21) that the ammonia production of Goldblatt kidneys is reduced furnishes further evidence in favor of diminished deaminization. On the basis of the experiments reported in this paper, the action of tyrosinase in reducing the blood pressure of hypertensive animals and man (22) is of particular interest, since this enzyme destroys phenolic compounds resembling hydroxytyramine in their chemical constitution and pharmacological action.

SUMMARY

Acute renal hypertension is produced by the injection of the amino acid dopa (*L*-dihydroxyphenylalanine) into the partially or completely ischemic kidney of the cat.

Evidence is presented suggesting that the rise in blood pressure following the injection of dopa is caused by its conversion into hydroxytyramine, a pressor amine.

Kidneys with normal blood flow fail to transform dopa into a pressor substance.

The possible importance of this reaction in the etiology of Goldblatt hypertension is discussed

We wish to express our gratitude to Dr H S Simms for performing the ultrafiltration.

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EXPLANATION OF PLATE 13

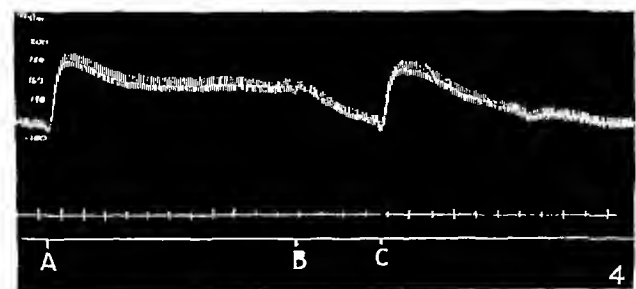
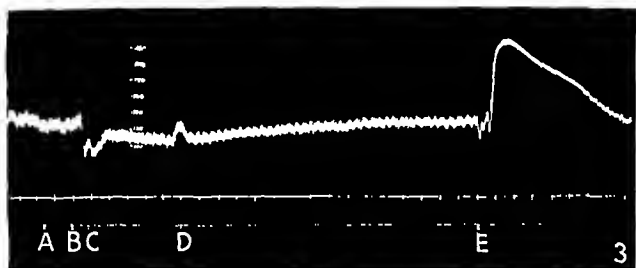
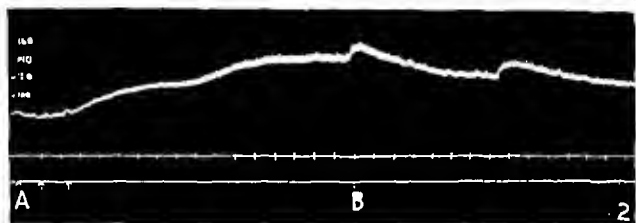
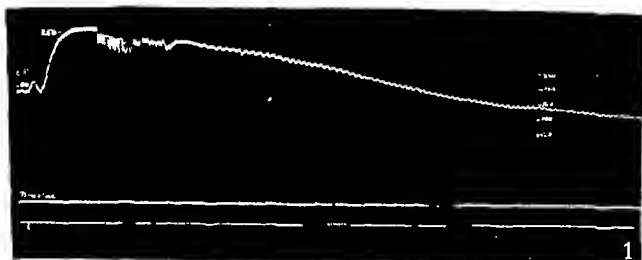
FIG 1 The effect of removing the serrefine from the left renal pedicle after 2 hours and 45 minutes of complete ischemia. Kidney contained 50 mg of dopa. The blood pressure rose from 170 to 255 mm Hg in 40 seconds. Time = 1 second.

FIGS 2 to 4 demonstrate the effect on the blood pressure of the injection of 10 mg of dopa into the partially ischemic kidney.

FIG 2 At *A*, the Goldblatt clamp was opened slightly. The blood pressure rose from 75 to 155 mm Hg in 16 minutes. At *B*, clamping of the left renal pedicle. The blood pressure fell to 110 mm Hg. Time = 1 minute.

FIG 3 At *A*, 10 mg of dopa were injected into the aorta. At *B*, the Goldblatt clamp was slightly opened. At *C*, the serrefines on the left renal vein and the aorta were removed. The blood pressure rose from 90 to 110 mm Hg. At *D*, the Goldblatt clamp was further opened. The blood pressure rose from 110 to 130 mm Hg. At *E*, the Goldblatt clamp was opened completely, causing the blood pressure to rise to 230 mm Hg. Time = 1 minute.

FIG 4 At *A*, the Goldblatt clamp was opened slightly. The blood pressure rose from 115 to 190 mm Hg. At *B*, the Goldblatt clamp was completely closed and the blood pressure fell to 120 mm Hg. At *C*, the Goldblatt clamp was opened moderately and the blood pressure rose to 180 mm Hg. Time = 1 minute.



IMMUNOLOGICAL SPECIFICITY OF MATERIAL SEDIMENTABLE AT HIGH SPEED PRESENT IN NORMAL AND TUMOR TISSUES*

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Attempts to isolate filterable agents producing sarcoma and leukosis led to the discovery that materials sedimentable at high speed are present in large amounts in normal tissues (1, 2). Claude (1), the first to call attention to the presence of such a material in non malignant tissue (chick embryo), presented observations indicating the similarity in the physical and chemical properties of the normal heavy material to that exhibiting virus activity, and suggested that the two are related. Since heavy fractions obtained from chicken spleen and chicken sarcoma could not be distinguished by complement fixation and precipitation reaction tests, it was suggested (2, 3) that the bulk of material obtained from tumors is normal heavy material which contains only a small amount of the tumor producing agent.

This opinion is supported by experiments indicating that the rabbit anti sera against chicken spleen do not neutralize the agent, while anti tumor sera possess strong neutralizing antibodies unrelated to the complement fixing antibodies.

Subsequent study has shown that heavy material is present in all normal and tumor tissues studied. If these materials are obtained from animal species in which this heterogenetic antigen is present (4) they contain tissue and organ specific substances (4, 5) and Forssman antigen. In preparations from heart muscle (6) they contain enzymes such as cytochrome oxidase and succinic dehydrogenase and in mouse kidney extracts phosphatase (7).

This heavy material seems to be a carrier of biologically active substances. Upon autolysis of mouse kidney phosphatase is liberated in an active non sedimentable form with a concomitant reduction in the amount of heavy fraction, at the same time the heterogenetic and tissue specific factors are destroyed (7).

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More recent experiments indicate that the Wassermann hapten is associated with the heavy material, which, however, does not function as a complete Wassermann antigen (8)

Kidd (9) found in extracts of non-filterable Brown-Pearce rabbit carcinoma a material that was sedimentable at high speed and fixed complement with sera of tumor-bearing rabbits. This complement-fixing antigen was not demonstrated in extracts from normal tissues, virus papillomas, and uterine cancers of rabbits. Thus, it differs immunologically from the heavy material found in normal tissues. It does not produce tumors when injected into susceptible rabbits (9).

The present study was undertaken to determine the immunological relationships of heavy materials present in organs of several animal species.

Material and Methods

Preparation of Antigen—Tissues were frozen at -60° and ground in the cold with sand and taken up in 2.5 to 5 times this volume of saline. The extract was centrifuged at 2000 R.P.M. and the supernatant fluid clarified by further centrifugation at 8000 R.P.M. for 15 minutes. These crude extracts were then recentrifuged at 27,000 R.P.M. for 1 hour, the supernatant was decanted, and the sediment washed with cold saline. The washings were discarded and the pellets suspended in saline. The insoluble material was removed by centrifugation in the cold at 2000 R.P.M., part of the supernatant solution was analyzed for nitrogen, and the rest stored at -60° .

Preparation of Immune Sera—Rabbits were injected intravenously four times weekly with alum-precipitated suspensions of high speed sediments. About 16 to 30 injections of suspensions containing 1 to 2 mg. protein per ml. were used for immunization. In several cases, the portion of the high speed sediment which failed to redissolve in saline was also used for immunization. Test bleedings were made 5 days after the last injection, and when the sera were satisfactory the animals were sacrificed and the sera kept in the ice box after adding merthiolate to make a final concentration of 1:10,000.

Antisera were prepared with heavy material from chicken tumor (Sarcoma 13 (10)), with spleen, and with chicken spleen heated to 100° for 1 hour, with human spleen and with human kidney, with mouse spleen and with mouse kidney, and with a suspension of leukemic mouse lymphocytes of a transmissible strain of mouse leukemia (Alf 5).

Complement Fixation Tests—Tests were made by both antigen and serum dilutions. Sera of low titer often reacted to high dilutions of the antigen. The antigens were standardized on the basis of their nitrogen content. The sera were titrated using an antigen concentration of 0.1 mg. N per ml., unless otherwise indicated. In all tests 0.2 ml. each of antigen and of serum were mixed with 0.2 ml. of guinea pig serum diluted to contain 2 units of complement. After the tubes were incubated at 37° for 1 hour and at room temperature for an additional hour, 0.2 ml. of a 5 per cent suspension of sensitized sheep cells was added and the tubes again incubated during 30 minutes at 37° and the degree of hemolysis determined. A second reading was

made after the tubes had remained in the ice box overnight. The presence of sheep hemolysins was determined by incubating mixtures of serum, complement, and a 2.5 per cent suspension of washed sheep cells for $\frac{1}{2}$ hour at 37°.

Correlation of the Amount of Heavy Material with Complement-Fixing Activity

Table I shows the distribution of complement fixing antigens in fractions of extracts of chicken tumor and of chicken spleen obtained by high speed centrifugation.

The clarified extracts of chicken tumor and spleen were centrifuged at 27,000 R.P.M. for 1 hour. The sediment was resuspended in saline, the insoluble debris removed, and the material recentrifuged at 27,000 R.P.M. The highest dilution of each solution which would give complete fixation of 2 units of complement was then measured and the amount of nitrogen (N) calculated.

TABLE I

Relation of Complement Fixation to Heavy Material Present in Extracts from Tumor and Spleen

	Tumor	Spleen
	mg N	mg N
A Crude extract	0.026	0.029
B Supernatant from A centrifuged at 27,000 R.P.M.	0.28	0.12
C Sediment, soluble fraction from A centrifuged at 27,000 R.P.M.	0.002	0.003
D Supernatant from C recentrifuged at 27,000 R.P.M.	0.06	0.14
E Sediment, soluble fraction from C recentrifuged at 27,000 R.P.M.	0.0015	0.0026
F Sediment, insoluble fraction	0.0027	

The anti tumor serum used (No. 24) had been absorbed with sheep cells and used in dilution of 1:100.

Approximately 0.2 ml. of a solution containing about 0.002 mg. of N per ml. will fix completely 2 units of complement (Table I). The minimum amount of nitrogen giving complement fixation is approximately the same with both spleen and tumor materials.

The Distribution of Forssman Antigen in the Heavy Materials Studied

Table II shows the distribution of Forssman antigen in heavy materials of mouse and of chicken organs as ascertained by complement fixation tests with antisera against heated high speed deposits from chicken spleen. Previous experiments have shown that heating to 100° destroys the tissue specific antigens but not the Forssman antigen. In these experiments Forssman antibody was kept constant (1:100 dilution of a strong immune serum against heated chicken spleen material) and the antigen was used in diminishing amounts.

Table II indicates the relative amount of Forssman antigen present in the tissues studied. Spleen, lung, kidney and tumor contain it in large quantities, while liver is devoid of Forssman antigen. Extracts from horse kidney and

guinea pig spleen gave as strong reactions as corresponding extracts from chicken and mouse tissues. Extracts from spleen and kidney of man, beef, and rabbit contained no Forssman antigen. Splenic extract from a man of blood group A gave a weak or doubtful reaction, splenic extract from a man of blood group O contained no Forssman antigen. Very large amounts of Forssman antigen were found in high speed deposits from venereal sarcoma of dogs.

Inhibition of hemolysis of sheep erythrocytes produced by the addition of substances containing the Forssman antigen, preceding the hemolysin test, as described by Brahn and Schiff (11), is as specific for the determination of Forssman antigen as direct complement fixation tests with Forssman antibodies (Table III).

TABLE II

Distribution of Forssman Antigen in Heavy Fractions from Organs of Different Species

	Spleen	Kidney	Liver	Heart	Lung	Tumor	Muscle	Testis	Brain	Leukemic in filtrates	Serum
Chicken	+++	++	0	+	+++	+++					0
Mouse	+++	++	0	++	+++	++	++	+	+	+++	

The calculations were based on a nitrogen content of 1 mg N per ml. Abbreviations: + = positive complement fixation with 1:10 dilution of this stock solution, ++ = positive reaction with from 1:30 to 1:50 dilution, +++ = positive reaction with greater than 1:50 dilution.

The alcoholic extracts were prepared as follows. The organs of animals were extracted with five volumes of 95 per cent alcohol at room temperature for 7 days. The extracts were centrifuged and concentrated to one-fifth of the original volume. The dilutions were prepared by adding the alcoholic extract to saline solution drop by drop until a 1:10 dilution was reached.

A preparation of hapten from horse kidney was kindly supplied by Dr. Karl Landsteiner.

The results of the inhibition with high speed deposits and alcoholic extracts were similar (Table III). No inhibition was found with the Wassermann antigen or with high speed sediment from rabbit spleen. In further studies of the antigenic properties of heavy materials, all antisera which contained the Forssman antigen were absorbed with washed sheep erythrocytes.

Immunological Relationship of Heavy Materials in Different Tissues of the Same Species

Table IV shows the relative amounts of heavy material which fixed complement with antisera against high speed deposits from chicken tumor and chicken spleen and with Forssman antiserum. The antisera against spleen and tumor

gave almost identical results. Heavy materials from heart gave slight, those from kidney and liver strong group reactions, spleen, lung, and tumor materials gave equally strong reactions. The liver contained no Forssman antigen, while the spleen and lung contained large amounts. Chicken serum or plasma in a wide range of dilutions failed to fix complement with any of the antisera against high speed sediments.

Table V shows the relative amount of heavy materials from mouse tissues fixing complement with antisera against high speed sediments from mouse kidney and spleen and with Forssman antiserum. The results suggest that

TABLE III

Specific Inhibition of Sheep Cell Hemolysis by High Speed Sediments and Alcoholic Extracts of Tissues Containing Forssman Antigen

Antigen	High speed sediments from				
	Chicken spleen	Chicken tumor	Guinea pig spleen	Rabbit spleen	Mouse spleen
mg N					
0.16	0	0	0	c	0
0.016	0	0	0	c	0
0.0016	c	ac	st	c	ac
0.00016	c	c	c	c	c
Antigen dilution	Alcoholic extracts from				
	Chicken spleen	Mouse spleen	Human spleen	Wassermann antigen	Horse kidney
1:10	0	0	0	—	0 (0.1%)
1:100	0	0	st	c	0 (0.01%)
1:1000	ac	ac	c	c	0 (0.001%)

Antiserum against heated chicken spleen containing 1.5 units of hemolysin and serum dilution 1:1000 were used. Abbreviations: 0 = complete inhibition, st = strong, ac = almost complete, c = complete hemolysis.

both spleen and kidney contain specific in addition to group reacting substances. Mouse liver, like chicken liver, contains no Forssman antigen in the dilutions tested.

Table VI shows the relative amounts of material sedimentable at high speed which react with antiserum against high speed deposits from human kidney, spleen, and liver.

The sera were prepared with tissues from men of blood group O and contained no hemolysins for sheep erythrocytes.

With the anti kidney serum, there is a strong cross-reaction between heavy materials from kidney and lung, while materials from spleen and heart give only slight or moderate cross-reaction.

TABLE IV

Complement Fixation Tests of High Speed Deposits from Chicken Organs with Antisera against Chicken Spleen and Chicken Tumor and with Forssman Antiserum

Antisera against heavy material from	Antigen N per ml	Heavy material from						Serum
		Tumor	Spleen	Kidney	Liver	Heart	Lung	
	mg							
Chicken tumor (No 24) absorbed with sheep cells	0 10	0	0	0	0	0	0	ac
	0 033	0	0	0	0	ac	0	ac
	0 011	0	0	st	st	c	0	ac
	0 0038	st	c	c	ac	c	sl	ac
Chicken spleen (No 26) absorbed with sheep cells	0 10	0	0	0	0	0	0	ac
	0 033	0	0	0	0	ac	0	ac
	0 011	0	0	c	c	c	0	ac
	0 0038	ac	st	c	c	c	tr	ac
Heated chicken spleen (No 45) (Forssman antibody)	0 10	0*	0	0*	ac	0*	0*	c
	0 033	0*	0	0*	c	0*	0*	c
	0 011	m*	0	st	c	ac	0*	c
	0 0038	c	0	c	c	c	0*	c

The sera were used in dilution 1 100

* Strong hemagglutination was noted in these tubes Abbreviations 0 = complete inhibition tr = trace, sl = slight, m = moderate, st = strong, ac = almost complete, c = complete hemolysis

TABLE V

Complement Fixation Tests of High Speed Sediments from Various Mouse Tissues

Antiserum against high speed deposits from	Antigen N per ml	Heavy material from							
		Kidney	Spleen	Liver	Heart	Lung	Muscle	Brain	Testis
	mg								
Mouse kidney (No 55) absorbed with sheep cells	0 10		0	c	c	0	c†	0	c
	0 02	0	0*	c	c	c		ac	c
	0 004	0	m†						
	0 0008	st							
Mouse spleen (No 35) absorbed with sheep cells	0 10	0	0	st	0	0	0†	0	sl
	0 02	tr	0	ac	st	sl	st	ac	ac
	0 004		ac						
Heated chicken spleen (No 45) (Forssman antibody)	0 10	0	0	c	0	0	0†	0	0
	0 02	sl	0	c	0	0	0	c	c
	0 004	ac	sl	c	c	ac	c		

* 0 033 mg N † 0 011 mg N ‡ 0 005 mg N

Serum 35 was used in dilution 1 75, the other two sera in dilution 1 100

With both anti spleen and anti liver serum there is strong cross-reaction with all materials tested

Absorption experiments were made to determine the character of the strong group reactions with the antisera against heavy materials from human liver. Kidney material was used for absorption because it reacted with this serum as strongly as the homologous substance. Human kidney was cut up into small particles, the fragments washed, mixed with immune serum, incubated at

TABLE VI

Complement Fixation Tests of High Speed Sediments from Various Human Tissues with Anti-Human Spleen and Kidney Sera

Antiserum against high speed deposits from	Antigen N per ml	High speed deposits from					Serum
		Spleen	Kidney	Liver	Heart	Lung	
	mg						
Human kidney (No 121)	0 10	tr	0	0	0	0	c
	0 033	tr	0	tr	0	0	c
	0 011	c	0	ac	ac	0	c
	0 0038	c	0	c	c	0	c
	0 0013		c			c	
Human spleen (No 67)	0 10	0	0	0	0	0	c
	0 033	0	0	0	0	0	c
	0 011	0	0	0	ac	0	c
	0 0038	sl	tr	c	c	0	c
	0 0013		c			c	
Human liver (No 76)	0 10	0	0	0	0	0	c
	0 033	0	0	0	0	0	c
	0 011	0	0	0	0	0	c
	0 0038	0	0	0	c	0	c
	0 0013		st	ac			

The anti kidney serum was used in dilution 1 100 the anti-spleen and liver sera in dilution 1 75

37° for 1 hour, and centrifuged. The partially absorbed supernatant was again mixed with washed kidney particles, incubated at 37° for 1 hour, and allowed to stand overnight in the ice box.

The absorbed antisera against human liver no longer gave complement fixation test with kidney material in any of the dilutions tested, while its reactivity with the homologous material was almost unaltered.

The anti kidney serum similarly absorbed with lung tissues lost most if not all of its antibodies reacting with lung and liver materials, but not those reacting with the homologous kidney substance.

The anti spleen sera on the other hand, lost both homologous and heterologous antibodies by absorption with lung tissue.

Search for Organ Specific Heavy Materials

Tests were made with sera at our disposal, to determine if some of the heavy materials possess organ specific substances Table VII shows that antisera against high speed deposits from chicken, mouse, and human spleen from

TABLE VII

Complement Fixation Reactions of Antisera against Heavy Fraction from Spleen with Splenic Antigens from Several Species

Antiserum against high speed deposits from	High speed deposit from spleen					
	Chicken	Mouse	Human A	Rabbit	Beef	Guinea pig
Chicken spleen (No 26) absorbed with sheep erythrocytes	+++	0	0	0	0	0
Mouse spleen (No 35) absorbed with sheep erythrocytes	0	+++	0	0	0	
Human spleen group O (No 121) unabsorbed	0	0	+++	0	0	

+++ = strong complement fixation 0 = no complement fixation

TABLE VIII

Complement Fixation Reactions of Anti-Kidney Sera with High Speed Sediments from Kidney of Several Species

Antiserum against high speed deposits from	Antigen N per ml	High speed deposit from kidney				
		Human	Mouse	Chicken	Rabbit	Beef
Human O kidney (No 121)	mg					
	0 10		m	c	st	c
	0 033	0	c	c	c	c
	0 011	0	c	c	c	c
Mouse kidney (No 56) absorbed with sheep cells	0 0038	0	c	c	c	c
	0 10		0	c	0	ac
	0 033	sl	0	c	0	c
	0 011	m	0	c	0	c
	0 0038	st	0	c	0	c

The sera were used in dilution 1 100

which the Forssman antibody had been removed by absorption with sheep erythrocytes reacted only with the homologous heavy materials

Table VIII shows that antiserum against human kidney is species specific, while the antiserum against mouse kidney gives strong reactions with high speed deposits from both mouse and rabbit kidney All three antisera against heavy material from mouse kidneys that were tested gave complement fixations with material from rabbit kidney The reaction of the same sera with human kidney material varied, two giving a slight and one no cross-reaction

DISCUSSION

Previous experiments have shown that high speed deposits from chicken sarcoma cannot be distinguished from similar material present in normal spleen, by either precipitation or complement fixation tests. Numerous experiments aimed to distinguish by immunological means malignant blood cells of mice from normal blood cells were likewise unsuccessful. In spite of these negative experiments, it seemed desirable to investigate further the immunological properties of heavy normal materials present in different tissues. Kidd (9) already has shown that certain rabbit tumors can be characterized by the presence of immunologically active heavy materials. It is possible that a better knowledge of the normal heavy materials will aid future studies aimed to demonstrate the presence of similar heavy substances in neoplastic tissues.

In the course of these investigations it became evident that the heavy material is one of the most significant components of the cell because it is associated with enzymes of the cell as well as with the Forssman antigen and the Wassermann hapten. A further correlation between the immunological and other properties of the cell and its heavy material is highly desirable. The observations thus far made indicate that the heavy material is complex and that the same material exhibits properties common to different tissues of the same species, to similar tissues of different species, and may also possess specificity characteristic only of one organ of a given species (4, 5). Studies on organ and individual specificities of whole tissues or tissue extracts by Witebsky (12) made by complement fixation tests and more recently by Bailey and Raffel using the technique of passive anaphylaxis (13), have yielded similar results. In the light of the investigations of Landsteiner and others on the specificity of immune reactions (14), these cross reactions and specificities can be attributed in part to similarities and differences in chemical structure. But there is no reason to suppose that the material obtained by high speed centrifugation is a single substance and some group reactions, for example that between materials from liver and spleen, are probably due to different antigenic substances. Attempts to separate the Forssman antigen from the tissue and organ specific components by cross precipitation absorption using Forssman antiserum and anti spleen serum, from which the Forssman antibody had been removed, were unsuccessful. This suggests that these specificities are not associated with two different substances, both are probably attached to the same heavy particle.

The question arose, whether the heavy material contains substances capable of producing cytotoxins. Experiments were made in association with Dr C. T. Olcott to determine if the high speed sediments from kidney extracts would produce nephrotoxins in animals. Both heavy material and antigens for nephrotoxins are present in the extracts of kidneys, both are antigenic, and differ from the antigenic substance of the plasma of the host. The observation that rabbits immunized with heavy materials from mouse kidney may develop

antibodies against heavy materials present in rabbit kidneys is of unusual interest. Unfortunately, the kidneys of these rabbits were not studied histologically and the few subsequent attempts made failed to demonstrate that antisera against the heavy materials from kidney possess nephrotoxic properties.

Recently, Henle and Chambers (15) obtained influenza virus unassociated with heavy materials by using allantoic and amniotic fluids of chick embryos inoculated with virus.

The question concerning the location of the heavy material in cells is of unusual interest. Claude (16) made observations suggesting that mitochondria are either carriers of the heavy material or are identical with it. Since both are of approximately the same size, the simple procedure of high speed centrifugation is not suitable to determine whether or not they are identical.

SUMMARY

Materials sedimentable at high speed (approximately 27,000 R P M) were found in all normal and neoplastic tissues studied. They are carriers of the Forssman antigen and of the Wassermann hapten.

These heavy materials exhibit species, organ, and individual specificity and produce several antibodies which can be demonstrated by absorption tests.

Heavy materials from chicken sarcoma and chicken spleen could not be distinguished by complement fixation tests.

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NEUTRALIZATION OF THE AGENT CAUSING LEUKOSIS AND SARCOMA OF FOWLS BY RABBIT ANTISERA*

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Shortly after the discovery that tumors and leukosis of fowls could be transmitted by cell free filtrates Rous and Murphy (1) observed that fowls in which tumors had regressed were resistant to subsequent inoculation, and that the sera of these fowls neutralized the transmitting agent. No precipitins or complement fixing antibodies could be demonstrated in these sera. Rous, Robertson, and Oliver (2) injected geese with tumor material and produced antisera which neutralized the agent. The complement fixing antibodies present in these sera were unrelated to the neutralizing activity, since absorption with erythrocytes from normal chicken sera failed to remove the neutralizing antibodies, but removed the complement fixing antibodies. These authors also immunized rabbits with tumor material and obtained hemolysins and hemagglutinins but no neutralizing antibodies. Twelve years later, Andrews (3) studied neutralizing antibodies produced in fowls against several different tumor agents and found that three histologically different filterable fowl tumors showed a close immunological relationship. By the use of pheasant antisera Andrews (4) could distinguish differences among several filterable fowl tumors although some cross neutralization occurred. Immune duck sera, however, did not show antigenic differences among the various strains studied.

Similar observations were made by Furth (5) and Uhl, Engelbreth Holm, and Rothe Meyer (6) with fowls recovered from leukosis. Recently, Ruffilli (7) described experiments suggesting that injection of fowl leukosis virus which had been inactivated by oxidation protects fowls against subsequent injection of the active agent. The immunity phenomena in fowl leukosis have been reviewed by Storti and Mezzadra (8).

Recently Andrews observed (9) that pheasants inoculated with a non filterable transmissible sarcoma induced originally by tar developed neutralizing antibodies against the Rous sarcoma agent and that antisera against fowl protein did not neutralize this agent. The neutralizing power of the serum against tar tumors was not affected by absorption with chick embryo pulp. These sera did not neutralize the agent of Fujimami sarcoma. Andrews concluded that this non filterable tar sarcoma contained a virus immunologically related to that of Rous Sarcoma I. This observation was confirmed by Foulds (10), who found that rabbit antisera against a non fil-

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terable transmissible tumor induced in fowls by 1 2 5 6 dibenzanthracene would neutralize the Rous agent

Recently it has been shown (11-13) that the agents causing fowl sarcoma and leukosis could be concentrated by the ultracentrifuge. The bulk of the sediment from tumor tissues, however, does not consist of virus, since large amounts of material can be sedimented at the same speed from normal tissues as well. Moreover, the materials obtained at high speed from chicken tumor and from normal chicken spleen were identical in complement fixation and precipitin tests using sera of rabbits that had been injected with these fractions (13). Studies on the purification of the agent of leukosis and sarcoma of fowls (strain 13 (14)), made it desirable to search for methods for differentiating the agent from the normal tissue protein. The experiments to be described show that virus-containing heavy materials induce the formation of neutralizing antibodies in the rabbit and that these antibodies are distinct from the complement-fixing antibodies. Similar sediments from normal chicken spleen produce no neutralizing antibodies.

EXPERIMENTAL

Heavy materials were prepared from extracts of normal chicken spleen and from tumors produced by strain 13, as previously described (13). Rabbits were inoculated intravenously four times weekly with alum-precipitated sediments of these materials, using a total of 15 to 40 mg of protein in 16 to 30 injections. Bleedings were made 5 days after the last injection. Sera were inactivated and filtered through a Berkefeld filter and stored without preservative under sterile conditions. Complement fixation tests were performed using 0.2 ml of antigen (0.10 mg N per ml), + 0.2 ml of the varying dilutions of serum, + 0.2 ml of guinea pig complement diluted to contain approximately 2 units of complement. After incubation for 1 hour at 37° followed by 1 hour at room temperature, 0.2 ml of a 5 per cent suspension of sensitized sheep erythrocytes was added to each tube. Hemolysis was read after ½ hour at 37°C.

Crude tumor extracts were employed in the neutralization tests, since they were more stable than the purified preparations. The tumor was ground with sand and saline, centrifuged to remove debris, and the viscous solution warmed to 37° with addition of enzyme preparation from pneumococcus (13) to reduce the viscosity. The solution was then filtered through a Berkefeld filter and stored in small tubes frozen at -60°C. Using the same extract, the neutralizing potency of several sera could be compared.

In carrying out the neutralization tests a measured volume of tumor extract was mixed with serum or saline and incubated at 37° for 15 minutes and allowed to stand overnight in the ice box. These mixtures were then diluted and injected into the breast and leg muscles of Barred Rock chicks of from 2 days to 1 month of age. By injecting the right breast and leg with one mixture, and the left breast and leg with another, different antisera could be compared in the same chickens.

RESULTS

The results of the neutralization experiments are summarized in Table I which shows that the rabbit antisera against heavy material from tumor neu-

TABLE I

Neutralization of Agents 13 and 11 with Rabbit Antisera against Heavy Materials from Chicken Tumor and Spleen

Antiserum	Tumor extract		Ratio of tumors produced to number of sites inoculated		
	ml	ml			
Experiment 1 Sarcoma 13					
Dilution of mixture			1 2500	1 250	1 25
Saline	2 4	0 1	9/10	8/10	9/10
Spleen 49	1 9	0 1	5/6	8/8	9/10
Experiment 2 Sarcoma 13					
Dilution of mixture			1 20 000	1 1000	1 50
Saline	2 45	0 05	0/10	3/10	8/8
Spleen 49	2 45	0 05	0/10	4/10	8/8
Tumor 70 ₁	2 45	0 05	0/10	1/10	4/8
Experiment 3 Sarcoma 13					
Dilution of mixture				1 1000	1 50
Saline	2 45	0 05		6/10	8/8
Spleen 48	2 45	0 05		5/10	6/10
Tumor 70 ₁	2 00	0 05		0/10	0/10
Tumor 71	2 45	0 05		0/10	0/8
Experiment 4 Sarcoma 13					
Dilution of mixture			1 5000	1 1000	1 60
Spleen 48	2 5	0 05	3/8	3/10	10/10
Tumor 71	0 1	0 05	4/10	3/10	8/10*
Tumor 71	0 5	0 05	0/10	1/10	5/10†
Tumor 71	2 5	0 05	0/8	0/10	0/10
Experiment 5 Sarcoma 13					
Dilution of mixture				1 1000	1 60
Spleen 49	2 5	0 05		3/8	8/10
Tumor 70 ₂ absorbed with spleen cell suspension	2 6	0 05		0/8	0/10
Tumor 70 ₂	2 5	0 05		0/10	0/10
Tumor 70 ₂	0 5	0 05		1/10	0/10
Experiment 6 Sarcoma 13					
Dilution of mixture			1 5000	1 1000	1 60
Spleen 48	1 0	0 06	0/8	6/10	8/10
Tumor 73	0 10	0 06	0/10	0/10	0/10
Tumor 73	0 50	0 06	0/10	0/10	0/10
Tumor 73	2 50	0 06	0/10	0/10	0/10
Experiment 7 Sarcoma 11					
Dilution of mixture			1 5000	1 500	1 50
Spleen 48	2 4	0 10	0/10	4/10	6/10
Tumor 71	2 4	0 10	0/10	0/10	4/10
Experiment 8 Sarcoma 11					
Dilution of mixture			1 2500	1 250	1 25
Spleen 49	2 4	0 10	2/10	5/10	7/9
Tumor 70 ₂	2 4	0 10	0/10	1/10	1/10

The total volume of the mixtures in Experiments 1, 2, 7 and 8 was 2.5 cc. in other experiments 3 cc.

* Large tumors

† Small tumors

tralize the agent. Antisera against heavy material from normal chicken spleen do not contain these neutralizing antibodies. The strength of the neutralizing antibodies in different sera can be determined by titration, using various amounts of immune serum with a constant amount of virus.

The neutralizing antibodies in the anti-tumor sera are unrelated to the complement-fixing antibodies. This is indicated by the observation that serum 71 contained neutralizing antibodies, but no complement-fixing antibodies. The complement-fixing antibodies in serum 70 could be removed by absorption with a suspension of cells from normal chicken spleen without any detectable effect on the neutralizing potency of the serum. Moreover, antisera against heavy materials from normal spleen do not neutralize the agent but fix complement in high dilutions. Thus, injection of heavy materials from tumor may give rise to antibodies specific for the agent, to complement-fixing antibodies against chicken tissue, or to both. It is noteworthy that sera of chickens immune to the viruses of leukosis and tumors contain neutralizing (3, 5, 6) but no complement-fixing antibodies (13).

Under the conditions of these experiments the neutralizing antibodies themselves do not fix complement. This may be either because the neutralization test will detect smaller amounts of antibody than the complement fixation reaction or because the neutralizing antibodies are unable to fix complement. Instances of the latter are well known, the antityphoid (H) antibodies to the typhoid bacillus in rabbits (15) and antipneumococcus horse serum (16) are outstanding examples. An instance of the former has recently been observed by Kidd (17) who found that most antisera against the Shope papilloma virus contained virus-neutralizing and complement-binding antibodies in the same relative proportion, but a few sera neutralized small amounts of virus, yet failed to bind complement.

These observations furnish additional evidence that the agent is only a small part of the heavy material obtainable from tumor tissue and that preparations of the agent hitherto regarded by several investigators as pure contain large amounts of normal heavy materials.

The observations of Amies (18) are not in agreement with this conclusion. Amies obtained by repeated fractional centrifugation a suspension of the agent which was apparently free from fowl protein and could be agglutinated specifically by sera of fowls bearing the corresponding tumor. The sera also contained neutralizing antibodies for the agent but hyperimmune rabbit anti-fowl sera also neutralized the agent. From this finding it is inferred that the tumor agent contains an antigen which is normally present in fowl tissue.

Experiments 7 and 8 show partial neutralization of agent 11 by antiserum against agent 13. This indicates some degree of serological relationship between agent 11, which causes only sarcoma, and agent 13, which has potentialities of producing both sarcoma and erythroleukosis.

SUMMARY

Neutralizing antibodies against fowl tumor agents can be produced in rabbits by injection of heavy materials obtained from chicken tumor. Similar sediments from normal chicken spleen produce no neutralizing antibodies. The complement fixing antibodies produced by both materials are unrelated to the neutralizing antibodies.

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FURTHER STUDIES OF THE INFECTIOUS UNIT OF VACCINIA

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In a previous communication (1) evidence was presented which was interpreted as indicating that, when vaccine virus was suitably introduced into the skin of a rabbit, the results of experiment were consistent with the hypothesis that a single particle (elementary body) of virus could give rise to infection. This conclusion rested in part upon knowledge of the physical characteristics of the virus and direct estimation of the number of elementary bodies comprising an infectious unit, but chiefly upon the results of applying certain statistical principles to the study. It has since been pointed out (2) that certain of the measurements we used were not fully satisfactory, and an alternative hypothesis has been presented (3), based on the assumption that host resistance plays the determining rôle in this as in other situations where a quantal response occurs. At the same time investigations of the problem in this laboratory have continued and a preliminary report of certain studies of a modified strain of virus has already been published (4). It is the purpose now to report fully on this modified vaccine virus, as well as to describe the results of a number of other experiments. Finally, these and other data will be considered in the light of the two hypotheses which have been put forward.

I Composition of the Infectious Unit of a Cultured Strain of Vaccinia

Previous studies of the infectivity of vaccine virus have been carried out with strains which had a reasonably high virulence for the rabbit. The advantages of using a virulent virus in ease of preparing material and ease of determining end points are evident. However, it is equally apparent that a complete statement of the mechanism of infection by a virus must include observations on strains of virus of low virulence. Accordingly, studies of the First Revived Strain (5) of vaccine virus, kindly made available to us by Dr T M Rivers, were begun soon after the completion of the previous study.

EXPERIMENTAL

Experiments were planned to give evidence on the probable composition of the infectious unit of this strain of virus in terms of elementary bodies. Efforts

pothesis is a tenable one. Applying this hypothesis, and assuming that at the 50 per cent dilution there are 0.69 particles¹ per unit inoculum of 0.25 cc, it would appear that the original suspension contained $10^{0.77} \times 4 = 0.69$ particles per cc. However, the data of Table I do not agree with this. The relation between dilution and number of lesions produced is again consistent with the hypothesis that a single particle is giving rise to a lesion, but on this assumption it would appear that each cubic centimeter of original culture contained $10^{1.74} \times 20$ particles of virus (inoculum of 0.05 cc). The discrepancy is considerably greater than any possible experimental error, and has been consistently demonstrated. Moreover, the ratio between chick and rabbit infectious units of virus is a reasonably constant one. It is perhaps easiest to explain the discrepancy by the assumption that particles of 2 sorts coexist in the suspension, one capable of infecting both chick and rabbit, and the other of infecting only the chick embryo.

This assumption is the one we were first inclined to accept (4), and was arrived at, apparently independently, by Gallagher and Woolpert (7) on the basis of their studies of a strain of vaccinia modified by passage through fetal rabbits. It has the merit of similarity to conditions known to exist among bacteria, in which virulent and avirulent members of a species may be carried together in culture for many generations. If this assumption be correct, regeneration of the rabbit virulent component should not be difficult, and further it should be possible to obtain the chick virulent strain in pure culture. Regeneration of the virus was first attempted.

Experiment 2—For the purpose of regeneration of the rabbit virulent strain, a rabbit was injected in each testicle with 1.5 cc. of whole culture. After 5 days, although no evident disease had appeared, the organs were removed, ground with sand, and made into a 10 per cent emulsion with Locke's solution. 1 cc. of this emulsion was then inoculated into a second rabbit, and the process was repeated once, 3 rabbits being inoculated in series. No disease appeared, and inoculation of chick embryonic membranes with centrifuged emulsion resulted in the appearance of no specific lesions. The experiment was repeated twice with similar results.

Since simple serial passage in rabbits had resulted only in loss of the virus, a second method was tried.

Experiment 3—In this experiment rabbit passage was alternated with culture *in vitro*. A rabbit was inoculated intratesticularly with whole culture and after 5 days the organs were removed. They were ground with sand and Locke's solution

¹ If it be assumed that 1 particle gives rise to a visible lesion, it can be shown by application of Poisson's binomial theorem that the suspension of particles giving rise to 50 per cent of positive results contains on the average 0.69 particles per unit volume, for $P = e^{-m}$ and for $P = 0.50$, $m = 0.69$.

to make a 10 per cent emulsion and 0.2 cc of this used to initiate a flask culture. After one subculture in flasks, the virus was returned to the testicles of a normal rabbit. This was repeated 8 times, and each time enough virus was recovered from the testicle to initiate growth in embryonic tissues, but not enough to survive a second rabbit passage.

This alternate rabbit and culture passage of virus failed to enhance the virulence of the virus sufficiently to allow successive rabbit passages. It was considered, however, that multiplication of the virus in the simultaneous presence of adult and embryonic cells might increase the rabbit virulence. This was tested as follows—

Experiment 4—Flask cultures were set up consisting of Tyrode's solution with 10 per cent of normal rabbit serum and an appropriate amount of minced tissue. This consisted of one half chick embryonic tissue and one half mouse kidney or rabbit kidney. It was first shown that medium containing mouse or rabbit kidney alone supported growth of the Board of Health strain of vaccine virus. The modified vaccine virus grew well under the conditions described and after 15 passages, 10 in chick embryo + mouse kidney and 5 in chick embryo + rabbit kidney subcultures were made into medium containing rabbit tissue alone. No multiplication occurred.

These various attempts to enhance the growth of the rabbit virulent strain of virus in the presumed mixture of strains or to enhance its virulence having failed, efforts were made to secure a "pure culture" of the chick virulent strain by taking advantage of the wide disparity in the relative concentrations of the 2 sorts of virus.

Experiment 5—A series of flasks of chick embryo Tyrode solution culture medium was prepared and seeded with culture virus. Three flasks were inoculated with 0.1 cc. of each of a series of dilutions from 10^{-2} to 10^{-8} of the last previous culture. After 5 days incubation each flask was tested for the presence of virus. The flasks inoculated with virus diluted 10^{-4} contained virus; those inoculated with the 10^{-5} dilution did not. It was assumed that the flasks containing virus had been seeded with a minimal amount; the amount actually introduced was much less than that required to initiate recognizable infection in a rabbit. From the virus-containing flasks a second series of dilutions of virus was prepared and medium seeded. Again virus appeared in large quantity in the flasks seeded with the 10^{-4} dilution, but none was present in those seeded with 10^{-5} dilution. The process was repeated once more. It was hoped that this serial culture of virus from a seed inoculum known to contain but a few chick infectious particles would rid the culture of rabbit infectious elements. The last culture when titrated in chick and rabbit gave these titers: estimated number of chick infectious units per cc. $10^{6.1}$, estimated number of rabbit infectious units per cc. 10^{-1} , difference, $10^{6.0}$.

It is evident from this experiment that a few chick infectious particles may give rise in culture to large quantities of virus, and that this virus when tested

has the same ratio between chick and rabbit titers as the initial material. It is evident then that the viral particles are essentially homogenous as far as this test is concerned.

RÉSUMÉ

Earlier studies of the First Revived Strain of vaccine virus seemed to indicate that infection in rabbits could follow the introduction of a single particle of virus. It was soon learned that many more viral particles were present than were infectious in the rabbit, and it was therefore assumed that particles of 2 sorts coexisted in the suspension. This conclusion was also arrived at by Gallagher and Woolpert (7), and the possibility is implied by Bryan and Beard (3) although with no experimental support. Attempts to favor the growth of the rabbit virulent component of the culture were unsuccessful, as were also attempts to free the chick virulent of rabbit virulent particles by using very dilute viral suspensions as seed inocula in cultures. These observations seemed to cast serious doubt on the first hypothesis and to suggest that the viral particles were essentially homogenous, a result requiring an entirely different concept of the mechanism of infection in the rabbit.

II The Comparative Virulence of Vaccine Virus for Various Hosts

In the experiments outlined above a striking difference in virulence of vaccine virus for 2 different hosts was demonstrated. While it has long been known that not all animals are equally susceptible to all strains of a given virus, it seemed worth while to investigate the infectivity of a number of other strains of vaccinia for several hosts, applying quantitative methods as exact as conditions permitted.

EXPERIMENTAL

It was proposed to make simultaneous titrations of a number of strains of vaccine virus of widely differing virulence in a number of hosts, *viz*, rabbit (intradermal inoculation), mouse (intracerebral inoculation), chick embryo (chorio-allantoic membrane), and guinea pig (intradermal inoculation).

Virus—Virus of various sources was used, as follows—

(a) Board of Health Strain (B H). Originally derived from the New York City Board of Health strain of virus, this strain has been propagated for several years in the testicles of rabbits. Subsequently it has been propagated by dermal inoculation.

(b) First Revived Strain of vaccine virus (C V I), derived from (a) and described in Part I above.

(c) Second Revived Strain of vaccine virus (C V II), derived from (b) by serial rabbit passages to enhance its virulence for this animal. Passages were made before the virulence had declined to its present level (5).

(d) Western Reserve Strain (W R), derived from (a) by 18 passages in mice by intracerebral inoculation (8)

(e) International Health Division (I H D) A strain of virus presumably derived originally from the B H strain, but propagated for many passages by intracerebral inoculation of mice, and kindly made available to us by Dr J E Smadel

(f) Connaught Laboratories (C L) Probably derived originally from the New York City Board of Health strain it has been passed for many generations in rabbit skin, using elementary bodies as seed

(g) Ohio State University—I (O S U) Originally a Lilly strain of vaccinia, propagated first by calf inoculation, and then on chick embryonic membranes, it was passed in fetal rabbits by Gallagher and Woolpert (7) with marked change in its virulence for adult rabbits It was kindly made available to us by Dr E B Adams and was received in its twenty fifth fetal passage

(h) Noguchi Originally a dermal strain of vaccinia, this was adapted to rabbit testicular propagation by Noguchi (9) It has since been carried by rabbit testicular passage

Animals—Rabbits and chick embryos were selected and used as in the experiments of Part I Large, white guinea pigs were used in order to facilitate the reading of skin lesions Swiss mice weighing 15 to 25 gm were secured from a single dealer

Titration of Virus—As end point for all titrations the 50 per cent dilution was selected, with a theoretical inoculum of 10 cc When intradermal titrations were made, a macroscopic lesion appearing after a latent period and present for 2 days was recorded as positive Death of the animal was considered a positive result in the intracerebral titrations since in the case of mice it was not considered possible to make sufficiently accurate clinical diagnoses of illness Discrete lesions on the chick allantoic membrane were enumerated, and the results were adjusted on the assumption that 0.69 particles were present per unit inoculum when the 50 per cent end point was computed Accordingly the figures obtained with chick membrane were divided by 0.69 before inclusion in the tables

A number of titrations of each virus were made in the available host animals

As far as possible parallel inoculations were made at the same time from the same set of dilutions of virus Thus a single series of dilutions of B H virus was divided into 3, and inoculations made intradermally in rabbits intracerebrally in mice and into embryonic membranes within 2 hours At least 4 inoculations were made at each dilution usually 5 or more Membrane counts were based on at least 6 satisfactory membranes and usually on 12 including membranes inoculated with 2 or 3 dilutions

The results of these titrations are presented in Table II To facilitate comparison the rabbit intradermal titer has been taken as standard, and titer in other hosts given in relation to this A titer lower than that in the rabbit is expressed by a positive, a titer higher than that in the rabbit by a negative logarithm Inspection of the table indicates that wide differences exist between the apparent titer in different hosts Taking as a series the rabbit mouse-chick embryo results, there appears to be a regular gradation in virulence

in the order $IHD = WR > BH > CVII > CVI$, expressed in the increasing amount of virus required to produce infection. Without final hypotheses as to the ultimate composition of the infectious unit, it is apparent that an inoculum producing disease in a given host contains a definite amount of virus. If then another host be inoculated, and require for the production of disease 100 times this viral concentration, it is reasonable to conclude that so far as the second host is concerned the virus is less virulent. In comparing the rabbit and mouse titers, it is seen that the WR strain gives only slightly higher titers in the rabbit skin than in the mouse brain. The original BH virus is definitely less virulent for the mouse, and with the CVII strain the difference

TABLE II
Titration of Vaccine Virus in Various Hosts

Strain of virus	Comparative titer (logarithm)*		
	Mouse (intracerebral)	Chick embryo	Guinea pig (intradermal)
WR	0 54		
IHD	0 54	0 56	2 97
BH	1 15	-0 53	2 45
CVII	3 42	0 32	2 48
CVI	-†	-4 83	—
OSU	—	—	3 10
Noguchi	-0 41	1 10	2 30
CL	5 30	0 33	1 55

* Figures represent logarithm of titer in rabbit skin minus log of titer in specified host All reduced to a theoretical inoculum of 10 cc

† No lesions with highest concentration of virus tested

becomes impressive. The CVI strain by our criteria is avirulent for mice in concentrations we were able to secure.

The difference in virulence is expressed further in the character of the reaction, and in the type of the titration curve. In Figs 2 and 3 are presented titration curves of WR and CVII strains, respectively, in mice. While the first departs definitely from the Poissonian curve, the second bears almost no resemblance to it. It appears that in addition to the requirement of greater quantity of virus to cause infection, other factors are operating, presumably to make the infection which does occur a milder one and allow host factors which are of minor importance in relation to a highly virulent virus to operate more effectively, thereby allowing difference between individual animals to become more apparent. This presumption is supported by data from the rabbit. The vaccinal lesions of BH strain resemble those of WR strain infection. Those of CVII strain are characterized by almost complete absence of massive necrosis, and no evident necrosis at all occurs with CVI strain.

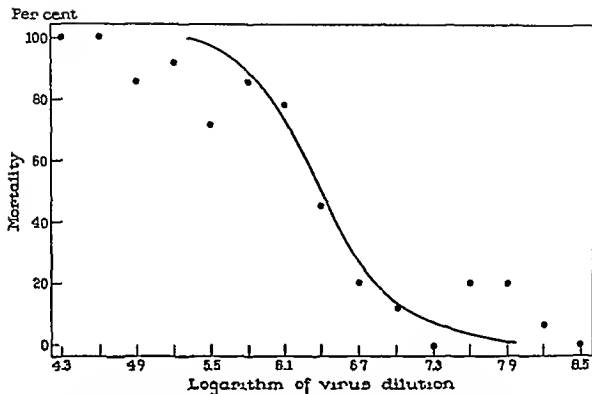


FIG 2 Titration of W R strain of vaccinia in the mouse. Mortality per cent is percentage of mice succumbing within 14 days after intracerebral inoculation

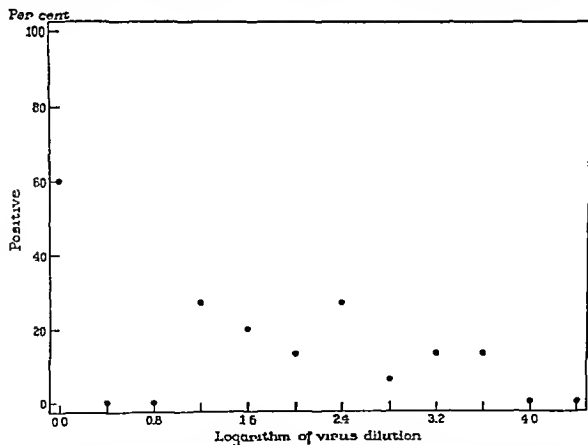


FIG 3 Titration of C V II strain of vaccinia in the mouse. Per cent positive represents the number of mice dying within 14 days after intracerebral inoculation

infection although examination with the microscope would probably reveal death of many individual cells. A similar tendency toward change in shape of the titration curve exists as was seen when virus was titrated in the mouse. With B H strain, or strains of similar virulence, no difficulty exists in demonstrating reasonably close correspondence between experimental and Poissonian curves when titrations in several rabbits are summed. With C V II strain the agreement is less good, and with C V I strain some difficulty exists in showing the relation. While the data on C V I strain presented in Part I do not repre-

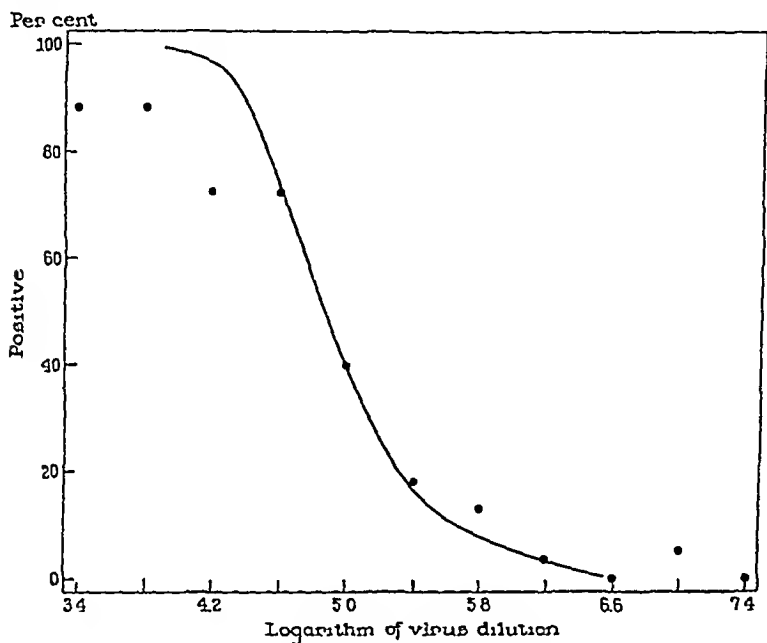


FIG 4 Titration of C V II strain of vaccinia in rabbits by intradermal inoculation

sent the results of unique experiments, most efforts to demonstrate the typical titration curve by summing the results obtained in several rabbits have failed. There are some data to support the possibility that results with this strain are more consistent when young animals are used for titration. In the case of C V II strain, it was pointed out (3) that the curves originally published do not fit the Poissonian distribution exactly and subsequent experiments have confirmed this. Results of such an experiment are presented in Fig 4. As before, the change is in the direction of flattening of the curve, a characteristic which will be discussed in greater detail later.

The figures in column 4 (Table II) indicate that in general guinea pig titer is much below rabbit titer of the strains observed. They do not suggest any

striking gradation of virulence between the strains. That such a difference did exist was evident from observation of the character of the lesions. Those due to Noguchi virus characteristically proceeded to necrosis involving most of the original area of the lesion. Necrosis occurred following infection with B H strain but was much less extensive, while little if any followed inoculation with C V II or O S U strains. With all of the strains positive opinions as to the presence or absence of lesions due to the more dilute inocula were difficult to arrive at, the nature of the guinea pig skin probably contributed to this. Variation was observed between animals, but as with rabbits the range of dilution between that producing consistently positive and negative results was not much in excess of tenfold to fiftyfold. Exact observations were not attempted.

That the "attenuation" of these strains of vaccinia for the mouse first and then the rabbit does not represent a pure loss as far as the virus itself is concerned is shown by the results of chick embryonic inoculation. If ability to initiate infection in the passage host be taken as standard, no loss of virulence has occurred. More, an actual increase has been produced, for the lesions produced by C V I strain are larger and more diffuse than those of B H strain and whereas heavy inoculation with B H strain produces in 48 hours a membrane with confluent lesions, inoculation with C V I strain in similar amount produces almost always a dead embryo. Because of this, few membranes are seen with confluent lesions, and even with smaller inocula few embryos survive to the 3rd day.

RÉSUMÉ

Vaccine virus of several strains has been titrated in several hosts, and the results have been presented. Taking end titers as indices, it is possible to arrange the strains in an order of virulence for mouse and rabbit with a mouse brain adapted strain (W R) first, then the original calf rabbit strain (B H), a revived cultured strain (C V II), and finally a degraded culture strain (C V I). When titration curves are determined for each host a similar order is evident, although the data are not so exact. It is shown that while mouse and rabbit virulence are declining in the cultured strains, an enhancement of virulence for the passage host (chick embryo) is occurring and that the C V I strain, almost avirulent for the rabbit, is highly virulent for the chick embryo. In terms of end titer all of the strains of vaccinia (which were primarily rabbit strains) were markedly less virulent for the guinea pig, although no such marked gradation in virulence was observed in this respect as has been noted above. Marked differences in the severity of the lesions produced were observed.

III Statistical Considerations in Determination of the Infectious Unit

In Parts I and II of the present study, data were presented bearing on the infectivity of various strains of vaccinia for several hosts, and attempts to

separate single strains from a presumed mixture of strains of virus were described. It was shown that, both with regard to the end-titer of suspension and character of titration curve, gradation of virulence can be demonstrated when several strains are studied. It is proposed in the present section to consider these and other data in light of the hypothesis originally proposed that a single viral particle under the proper circumstances can give rise to infection.

It has been pointed out by Haldane that the commonly employed method of computation of χ^2 which we used to determine the goodness of fit of our curves was not fully adequate, and he suggested the use of a more accurate method. Application of this to the data for one strain of virus indicated that the hypothesis was a tenable one. Bryan and Beard (3) applied the method in a study of the virus of rabbit papilloma, as well as vaccinia, and pointed out that the proper calculation of goodness of fit for the data which we presented for other strains of vaccine virus indicated that for these strains the hypothesis which we accepted was not tenable, an observation which we have confirmed. They pointed out, moreover, that if the data for vaccinia were treated on the assumption that viral distribution was unimportant, but that the response of the rabbit skin was variable, and that the degree of resistance was normally distributed in terms of viral dose, a good agreement existed for all of the strains of virus between hypothesis and experiment. On this basis, they were led to conclude that the variation in response to small doses of virus was an expression simply of variable susceptibility of different rabbits, and of variation in susceptibility of different sites on the same rabbit. They showed that good fit between hypothesis and experiment could be obtained on the assumption that the degree of resistance to infection was normally distributed, applying here the technique of Gaddum (10).

EXPERIMENTAL

In this section it is proposed to reexamine certain already published data, and also to present the results of experiments designed to give information on the question of the possible rôle of host resistance in determining the infectious unit of vaccinia.

Materials and Methods

The experimental methods, strains of virus, and animals have been fully described in Parts I and II of this communication. Relevant details will be added in connection with individual experiments.

It was shown above that the infectious unit of virus for one host might represent a thousandfold multiple of the amount of virus which comprised an infectious unit for another host. Further, it appeared that as the virulence of a viral strain for a given host decreased, the titration curve in that host tended to depart more and more from the simple binomial expansion, but that even with a virus of low virulence for the rabbit, it was still possible to demonstrate that infection appeared to follow the introduction of a single viral particle. A definite departure from theory was found with the second revived strain of

culture virus, and an experiment was performed to gain more detailed information

Experiment 6—A suspension of virus of the C V II strain was freed of gross particles and clumped elementary bodies by strong centrifugation. Preliminary titration indicated a titer on rabbits of $10^{-4.5}$ and for accurate titration, therefore suspensions were prepared in dilutions from $10^{-3.0}$ to $10^{-7.2}$, at intervals of $10^{-0.4}$. Rabbit intra dermal inoculations were made in amounts of 0.1 cc., and 10 inoculations of each suspension made in each of 6 rabbits

TABLE III
Results of Titration of C V II Strain of Vaccinia Simultaneously in Several Rabbits

	Dilution of suspension (logarithm)	Results of inoculation of individual rabbits													
		4-79		4-81		4-80		4-82		4-83		4-84		Total	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
	3.4	10	0			10	0	4	6	10	0	10	0	44	6
	3.8	10	0	10	0	10	0	4	6	9	1	10	0	53	7
	4.2	6	4	10	0	10	0	0	10	9	1	8	2	43	17
	4.6	10	0	8	2	10	0	0	10	7	3	8	2	43	17
	5.0	4	6	7	3	7	3	0	10	3	7	1	9	22	38
	5.4	1	9	1	9	3	7	0	10	2	8	3	7	10	50
	5.8	0	10	2	8	2	8	0	10	1	9	3	7	8	52
	6.2	0	10	0	10	0	10	0	10	1	9	0	10	1	59
	6.6	0	10	0	10	0	10	0	10	0	10	0	10	0	60
	7.0	1	9	0	10	1	9	0	10	0	10	0	10	2	58
	7.4	0	10	0	10	0	10	0	10	0	10	0	10	0	60
50 per cent dilution		4.84		5.13		5.29		3.49		4.84		4.86		5.00	
χ^2		1.18		3.26		0.15		3.9		5.3		7.2		102.00	
n		3.00		3.00		2.00		2.00		3.00		2.00			
P		0.79		0.36		0.92		0.15		0.26		0.03		<0.001	

The "average" titration curves, representing percentage of inoculations positive and negative for each viral dilution, are given in Fig. 4 of Part II. The lack of agreement between theory and experiment is evident, and is confirmed by the χ^2 test ($P < 0.001$). In Table III the data are given for the individual rabbits. It was postulated that each rabbit might constitute a separate universe in relation to the virus and the data have therefore been treated as 6 separate experiments. Inspection of the bottom row reveals in most cases a reasonably good agreement between theory and data. P for goodness of fit to the original Poissonian curve ranges from 0.03 to 0.92 and in no case is the value low enough to indicate that the hypothesis is certainly not tenable. For 5 of the 6 the P of 0.15 or more indicates reasonably good agreement.

The logical conclusion from this experiment would seem to be that, while a titration curve constructed for this strain by pooling results obtained with several animals is not a binomial one, the data for each rabbit which contribute to the total do follow the binomial expansion. It appears, moreover, that as the virulence of the virus increases, the summed curve tends to approach the binomial expansion as the limit. Additional evidence for this is obtained from physical and chemical studies.

Bryan and Beard pointed out that published data did not support the concept that one elementary body comprised an infectious unit. Reexamination of the papers quoted by them does not support their interpretation. Thus according to McFarlane's data (11) 1000 elementary bodies would be needed for an infectious unit (12*a*), however his preparations were apparently highly impure (12*b*). The figure arrived at by Sprunt, Marx, and Beard was 366 elementary bodies per infectious unit (13). This was based on physical characteristics determined by others, with different strains of virus, and the authors themselves gave very scanty data on the methods for purifying the suspension. They referred likewise to the figure of 42 elementary bodies per infectious unit based on work of Parker and Rivers (14). The preparations made in that study were not microscopically pure, as was pointed out at that time, although examination of the stained sediment did reveal a "minimum of extraneous material." Later intensive work on the physical and chemical constitution of vaccinia has continued to yield much information, and methods of purification have progressively been refined in that laboratory. The most recent relevant publication (12*b*) indicates a ratio of 42 elementary bodies per infectious unit, calculated from data on size and density obtained in the same laboratory. Further, the purity of the suspensions was checked and confirmed by ultracentrifugal analysis.

If the logic long honored in chemical research is applied to the data cited above, the conclusion seems inescapable that as purer suspensions of virus are obtained, the number of elementary bodies per infectious unit for virus of high virulence approaches 1, and the fact that with impure preparations it is higher substantiates not vitiates this conclusion.

As was noted in Part II the infectious unit of vaccine virus for the guinea pig contained many rabbit skin infectious units, and likewise some difference was observed in the reactivity of single pigs. While conditions of space as well as difficulties in exact classification of reactions precluded exact measurements, it was evident that the character of the titration curve for the individual pigs was not inconsistent with a binomial distribution of lesions. Similarly with C V I strain in rabbits, the infectious unit comprised many chick infectious particles. It was shown that with C V II strain, while the distribution calculated from the sum of several rabbit titrations did not follow a Poissonian distribution, the titration curve of each individual rabbit very definitely did. It appeared important, therefore, to reexamine the results obtained with C V I strain.

Experiment 7—A large volume of culture of C V I strain was prepared free of tissue, and concentrated by centrifugation. The virus was in its fifty third culture passage in this laboratory. Titration in chick embryonic membranes indicated a concentration of chick infectious particles of $10^{8.1}$ per cc. From the concentrated suspension twofold dilutions were prepared, and inoculated into rabbits in 0.1 cc. amounts, 10 inoculations per rabbit per dilution. The rabbits were observed daily, and presence or absence of lesions at the site of inoculation was recorded. As before, difficulty was experienced in classifying the results with complete assurance, the data accepted represent the opinion of 2 observers. They are presented in Table IV.

TABLE IV
Titration of C V I Strain of Vaccine Virus in Rabbits

Dilution of suspension (logarithm)	Rabbit No						Total
	5-76	5-77	5-78	5-79	5-80	5-81	
0 0	10*	10	10	10	10	8†	58
0 3	10	10	10	9	10	10	59
0 6	10	10	10	7	10	10	57
0 9	10	8	9	5	10	10	52
1 2	7	9	7	1	10	10	44
1 5	0	6	0	0	9	0	15
1 8	0	0	0	0	0	0	0
2 1	0	0	0	0	0	0	0
2 4	0	0	0	0	0	0	0
2 7	0	0	0	0	0	0	0
50 per cent dilution	1 29	1 47	1 26	0 72	1 63	1 35	1 32
P (1 particle)	<0 001	<0 001	<0 001	0 48	<0 001	<0 001	
P (4 particles)		0 07					
P (10 particles)	0 22	0 01	0 31	<0 001	0 14	0 06	

* Number of inoculations positive of a total of 10

† Total of 8 inoculations of this dilution

Inspection of Table IV reveals marked disparity between theory and experiment for the summed titration curve. Separating this into its component curves does not help much, as inspection of the row designated *P* (1 particle) indicates. In one set (R 579) good agreement is indicated, in the others the hypothesis that a single particle causes infection is obviously untenable. When the data are reconsidered on the assumption that 10 particles are required to cause infection, 4 of the sets indicate a possibility of agreement with the hypothesis and the remaining set can be harmonized with the assumption that the number of particles required is intermediate.

RESUMÉ

The data presented in this part appear to indicate that with increasing virulence of a viral strain the titration curve derived from the results of inocula

tion of several rabbits tends to approach as its limit the Poissonian binomial curve, and reconsideration of published data on the physical characteristics of an infectious unit indicates that with virus of maximum virulence an infectious unit is contained in a single elementary body. As virulence declines, the infectious unit comes to contain more and more elementary bodies, and the titration curve departs more and more from its original form. When the loss of virulence is moderate, as it is with the C V II strain, examination of the titration on individual rabbits indicates that for a single animal the binomial expansion, using the assumption that single particle initiates infection, describes the findings adequately. The lack of agreement with theory of the summed curve is largely due to variation between rabbits. When virulence is almost completely lost, as it is in the C V I strain, not only does the summed curve tend to depart from the original one, but the data of the individual animals also tend to become discrepant. Here the curve does not have a decreased but a sharply increased slope, the tendency is in the direction which would be expected on the assumption that more and more particles are required to initiate infection. As with the other strain of virus, variation in susceptibility of rabbits is observed.

DISCUSSION

It was postulated by early workers in the field that the viruses require an intracellular location for growth. Subsequent work has tended only to support that conception, and it seems well established now that while the virus of vaccinia will survive *in vitro* in the absence of living cells, for multiplication it requires living cells, and some evidence is already available that it requires cells of a particular sort. Thus it was shown by Rivers (15) that in the cornea it was principally the young cells, growing in response to the stimulus of trauma, which are readily available for vaccinal infection. Further, the data of Part II strongly suggest that difference exists between embryonic and fully differentiated cells with regard to susceptibility to infections. Much supporting evidence for this conception might be gathered from characteristics of other viruses, e g, the predilection of the poliomyelitic virus for anterior horn cells, of louping ill virus for the monkey's Purkinje cells, of viscerotropic yellow fever for hepatic, and of neurotropic yellow fever for nervous cells.

Obligate parasitism connotes an inability of the parasite to survive without deriving energy from a more complete organism, and the intracellular habitat of viruses strongly indicates that the source of energy for the virus is located near the heart of the cell's own economy. If this be true, adaptation on the part of a virus would consist in development of those energy-producing systems which would function most efficiently in the presence of the particular conditions existing within the available cells. Cells are known to differ widely in their composition and energy-producing mechanisms, a difference which would

be expected to reach its full development with the maximum differentiation of the cell. It is not implied that such differentiated cells bear no physiologic resemblance to other fully differentiated cells of another sort, but it seems apparent that profound differences may exist, and it does not seem unlikely that virus well adapted to survival under one set of conditions, might find another intracellular situation so unsuitable for multiplication as to render successful parasitism impossible. Accordingly, with regard to the initial phases of viral infection each host is to be regarded not as a single animal, but as a universe of cells differing widely in internal composition, some suitable, some unsuitable for viral multiplication. The possibility that such a situation might exist was suggested by Sprunt and McDearman (16), who pointed out that the "probability of insertion" of a particle was really the probability that the inoculated particle reached a susceptible cell. A virulent virus then would be one capable of deriving energy from a system common to many cells. Loss of virulence might be the expression of general loss of "growth potential," but it might also be the expression of the development on the part of a viral strain of capacity to parasitize cells having one sort of make up at the expense of ability to parasitize a cell with different predominating energy systems. "Virulence" then has meaning only when both virus and host are exactly characterized. In developing this conception fully, it might be postulated that differences between individual animals with respect to viral infection are expressions of differences in the proportion of cells available and unavailable for parasitism. With a fully virulent virus, able to parasitize cells of a wide range, the probability of infection would be essentially the probability of insertion of a viral particle. With decline in virulence for a given host (perhaps in the course of adaptation to a different host) fewer and fewer cells would be available for parasitism, and 2 factors would then combine: the probability of inclusion of a viral particle in the inoculum, and the chance of a viral particle entering a susceptible cell. If but 1 host animal is available, no possibility exists of distinguishing between these possibilities on the basis of the results of inoculation. However, it is likely that with decreasing virulence and smaller number of cells available, individual animals will differ in the proportion of susceptible to resistant cells. Then, as far as the individual animal is concerned, the distribution of lesions in relation to viral concentration will remain the same, but combination of the data from several animals will no longer give a binomial distribution. To the binomial distribution obtained when the single animal is observed, has been added a normal distribution expressing the differences between animals. The data indicate that this is the case here.

It was shown that the difference in titer of C V I strain of virus as determined in 2 hosts was of the order of 10^5 , the hypothesis elaborated above would imply that the ratio of C V I susceptible cells between chick embryo and rabbit is of the order of $1-10^5$. If this be true it is difficult to visualize the way in which

infection, even if initiated, could be maintained, but in this circumstance other factors enter. It may be taken as axiomatic that only virus which enters susceptible cells can cause infection, but it must also be recognized that the viral particles do not necessarily have a predilection for entering such cells. In fact, examination of tissues after injection of identifiable particles shows that there is a marked tendency for the foreign material to be engulfed by cells of a particular type which have been variously designated, but may be described generally as phagocytes. Some of the material also enters lymphatic channels and is removed to a distance. It does not seem unreasonable to assume that the locally pathogenic action of viral particles carried away in lymphatics or engulfed by phagocytes may be reduced when not prevented completely. This leaves a proportion of particles, assumed to be constant for each animal, which are free to enter other cells of various sorts, and it is upon the chance that these cells are susceptible to viral growth that the chance of infection depends. Therefore, the infectivity ratio of $1-10^5$ does not necessarily mean that for each rabbit cell available to infection there are 10^5 unavailable, but simply that the chances of a particle entering a susceptible cell are 1 in 10^5 . To this must be added the possibility that the infection which might arise from parasitization of a single cell might not cause a tissue reaction of sufficient magnitude to produce that erythema and edema which are essential to a diagnosis of local vaccinal infection. The combination of several such foci, each arising from a single cell, might, however, cause visible signs of inflammation. The data of Experiment 7 are consistent with this postulate. It is not to be anticipated that the number of foci involved in different rabbits will be uniform, for aside from the chance of combination of foci, there is added the variation in reactivity of rabbits to a uniformly noxious agent. Likewise too much stress is not to be laid on the fact that good agreement is obtained between an experimental curve and a theoretical one deduced from the assumption that 5 or 10 or any other number of foci must be present to cause visible signs of infection. Many factors are operating, not all of which are known, to produce the observed distribution. The essential element is that the slope obtained is much steeper than expected for a 1 particle distribution, which is consistent with the hypothesis proposed, and which would not be expected to follow the operation of the usual random errors.

CONCLUSIONS

A study has been made of the comparative virulence of several strains of vaccine virus for a number of hosts, and wide variation in animal susceptibility has been demonstrated. The results obtained in experiments with a chick-embryo-adapted strain are interpreted as indicating that the particles of virus are of essentially uniform virulence. Results of statistical analyses are presented which indicate that as the virulence of a strain of virus increases the

number of elementary bodies per infectious unit approaches 1, and at that limit the chance of infection is governed primarily by the presence or absence of virus in the inoculum. With lower virulence the chance of a lesion following inoculation of virus is still described by the binomial theorem, but the actual distribution is primarily of susceptible cells not of viral particles. It is postulated that with regard to the proportion of cells available for parasitism, differences exist between different animals of a species, and that this distribution is of a normal character.

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ENDOCRINE FUNCTION OF THE SURGICALLY REDUCED PANCREAS

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The investigations of von Mering and Minkowski (1890) first demonstrated that it is possible to resect large portions of the pancreas without producing diabetes or glycosuria. Minkowski (1890, 1893) found that as little as one-quarter or one fifth of the pancreas suffices to control glycosuria. Allen (1913) observed that diabetes follows very rarely when one-sixth or one-seventh of the pancreas remains, with an eighth of the pancreas remaining diabetes is mild, if only one tenth remains the diabetes is pronounced, although in the experimental protocols there were cases without glycosuria in dogs in which only 2 to 3 gm of pancreas remained. Both Minkowski and Hedon (1898) found that very small portions of the pancreas prevent diabetes, although it was easier to produce alimentary glycosuria in depancreatized than in normal animals.

Allen (1913) obtained similar results. He demonstrated also that a rich carbohydrate diet may alter the function of the remaining pancreatic tissue rendering it insufficient and producing diabetes progressively pronounced. Confirming these results, it was found many years ago in this laboratory that a large sugar intake (50 to 100 gm daily), starting soon after the operation determined the appearance of progressively pronounced diabetes in the great majority of dogs having 2 to 3 gm of pancreas but very rarely in those having 5 to 6 or more gm of pancreas. It was observed also that only the total resection of the pancreas produces very pronounced diabetes with any certainty in healthy dogs that are properly fed. With subtotal resection it is not possible to predict what the immediate intensity of the diabetes will be. By leaving 0.9 to 1.6 gm of pancreas after resection in dogs of 9.8 to 13.5 kilos a mild diabetes (diabetes of Sandmeyer) was obtained which progressed to fatal termination (Quián, 1932, 1933). Three dogs weighing 7.3, 7.3 and 10 kilos left with 2 to 3 gm of pancreas and 6 dogs weighing 6.7 to 10 kilos left with 4 to 6 gm of pancreas maintained normal glycemia after operation. In only one of them was there glycosuria and a slight degree of hyperglycemia (Houssay, Biasotti and Rietti, 1932). It has subsequently been confirmed in a greater number of dogs that if 4 or more gm of the gland remain only exceptionally are hyperglycemia and glycosuria observed with 3 gm of pancreas about 50 per cent of the animals maintain normal glycemia without glycosuria and the other 50 per cent have mild diabetes which is progressive and results in death in cachexia.

In the first days following subtotal resection of the pancreas glycosuria frequently is observed. It may be produced by traumatic alteration and postoperative degeneration of the pancreas and subsequently disappear or remain. If small doses of insulin

(5 to 10 units per day) are injected for a period of 8 to 10 days the animals recover more promptly than those not receiving it. With insulin treatment it is possible to have animals with the smaller amounts of pancreatic tissue free from glycosuria and diabetes.

Dogs with reduced pancreatic tissue are very sensitive to the diabetogenic action of extracts of the anterior hypophysis. They develop hyperglycemia with smaller doses than do normal dogs and if the injection of the anterior hypophysis is continued for a prolonged period they may become diabetic and die in cachexia in a few days or weeks even though the injections are suspended (Houssay, Biasotti, and Rietti, 1932, Houssay, Biasotti, Di Benedetto, and Rietti, 1932, 1933). These experiments were the first demonstration of permanent diabetes elicited by injection of extract of anterior hypophysis. This diabetes remained after discontinuing the injections of the extract. The results have been confirmed on the cat by Dohan and Lukens (1939) and Lukens and Dohan (1940) and in the rat by Long (1937, 1939).

Since 1932 we have observed that a return of normal glycemia may be induced in animals diabetic from injection of anterior hypophyseal extract if the hypophysis is resected or if insulin is injected. The use of insulin promotes healing of the histologic lesions of the pancreatic island in the cat according to Lukens and Dohan (1940). Hypophysectomized dogs weighing 8 to 10 kilos having about 4 gm. of pancreas are very sensitive to the action of the extract of anterior hypophysis although the glycemia returns rapidly to the normal level soon after suspending injections (Houssay and Biasotti, 1938, Houssay, 1939).

Insulin content and histopathological methods have been used to appraise the endocrine function of the pancreas but a more direct physiological measurement is preferable. The present studies were undertaken to test the functional capacity of the pancreas with various degrees of surgical reduction and under such strains as result from intravenous glucose and injection of anterior hypophyseal extract. Further observations of this function were obtained by the use of the temporary (Gayet) graft of the altered pancreas in animals with complete pancreatectomy.

Experimental Procedure

We have made comparative studies of the function of the pancreas at rest and under conditions of strain in normal dogs, in dogs with the pancreas surgically reduced to about 10 gm., which is a reduction of approximately 50 per cent of normal, and in dogs with the pancreas reduced to about 4 gm., which represents a reduction to approximately 20 per cent of normal. In these animals some weeks after the healing of the operative wound determinations were made of (1) glycemia before and after meals, (2) the hyperglycemic curve produced by intravenous administration of 1 gm. per kilo of glucose, (3) the diabetogenic dose of anterior lobe of the bovine hypophysis, and (4) the insulin secretion of pancreas grafted by vascular anastomosis in the cervical region of the pancreatectomized diabetic dogs.

Methods—Dogs weighing 8.5 to 13 kilos were used, the great majority were 9 to 12 kilos and males. They were fed about 30 to 40 gm per kilo of raw beef given in one meal at 2 p.m. every day. In the morning fasting blood for glucose determinations was taken from an incision on the edge of the ear. The Hagedorn Jensen method for glucose determination was used after precipitation by the Somogyi method.

The tolerance to glucose was tested in non anesthetized animals injected intravenously with 1 gm of glucose per kilo of body weight in 33 per cent solution in

TABLE I

Intravenous Injection of 1 Gm. of Dextrose per Kilo without Anesthesia. Time in Which the Glycemia Returned to 120 Mg. Per Cent

Time of return	No. of animals				
	1 hr	1½ hrs	2 hrs	3 hrs	More than 3 hrs
Normal dogs	9	1	1	—	—
Dogs with pancreas reduced to 10 gm	2	3	1	—	—
Dogs with pancreas reduced to 4 gm	4	—	1	4	1

TABLE II

Dogs with Reduced Pancreas of 4 Gm. Hyperglycemic Curves after the Injection by Intravenous Route of 1 Gm. of Glucose per Kilo of Body Weight

Dog No	Weight kg	Days post operative	Blood sugar					
			Before Injection	15 min	1 hr	1½ hrs	2 hrs	3 hrs
			mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
5-5	12.0	16	106	299	120	103	99	96
5-6	12.0	16	81	287	190	170	140	108
5-4	10.0	20	84	252	116	101	—	97
6-1	11.0	20	101	258	181	154	123	93
6-2	9.5	20	83	284	195	154	139	97
5-2	7.0	20	107	335	211	180	151	122
5-0	8.5	24	117	306	194	146	137	110
7-2	5.0	54	113	282	115	90	83	81
7-3	10.0	32	92	233	110	96	87	83
7-5	6.3	29	110	313	207	138	119	98

distilled water. Samples of blood were taken before the injection of dextrose and 15 minutes, 1, 1½, 2, and 3 hours after. The animals received no food during the 19 hours before the injection and were kept fasting during the test.

Extract of the anterior hypophysis was injected intraperitoneally twice a day for 4 days. An extract of the anterior lobe of the bovine hypophysis in saline solution was used. The glands were frozen with carbon dioxide snow as soon as they were obtained shortly after the animals were slaughtered. The extracts were prepared at a temperature of 2–4°C and were kept frozen until the moment of the injection in order to avoid a decrease in potency in the diabetogenic hormone.

Injections of equal doses of anterior hypophysis extract were given daily for 4

days and then were discontinued for from 3 to 10 days. If the glycemic level of 150 mg per cent was not produced a larger dose was given the following week until the desired hyperglycemia was elicited. When the desired level was reached injections were discontinued for at least 10 days. The milligram dose per kilo per day injected

TABLE III

Dogs with Reduced Pancreas of 10 Gm Hyperglycemic Curves after the Injection of 1 Gm Glucose per Kilo of Body Weight by Intravenous Route

Dog No	Weight	Days post-operative	Blood sugar					
			Before injection	15 min	1 hr	1½ hrs	2 hrs	3 hrs
			mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
5	9 0	9	95	236	171	86	84	86
1-8	7 5	36	95	253	153	103	98	75
1-6	11 0	47	82	253	100	85	82	75
1-4	8 2	50	98	240	110	87	82	85
4	8 0	59	99	258	157	132	117	92
2	9 0	61	99	236	137	115	97	—

TABLE IV

Normal Control Dogs Hyperglycemic Curves after Intravenous Injection of 1 Gm Glucose per Kilo of Body Weight

Dog No	Weight	Blood sugar					
		Before injection	15 min	1 hr	1½ hrs	2 hrs	3 hrs
		mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
1	9 0	83	274	62	56	61	64
2	13 0	77	357	179	143	93	67
3	8 0	80	131	80	85	78	99
4	7 5	71	156	75	83	75	80
5	10 0	76	163	91	91	87	81
6	6 0	82	211	108	86	84	77
7	12 0	91	163	94	84	96	87
8	9 0	80	200	95	86	80	89
9	8 5	94	200	100	87	94	100
1-0	10 5	118	230	182	100	86	94
1-1	10 5	100	238	84	88	80	94

during these 4 days in each experiment was 20, 40, 80, 100, 150, 250, 500, 1000, 1500, or more until a positive effect was obtained.

The capacity to secrete insulin as determined by the effect on diabetic hyperglycemia was studied by grafting the pancreas in the neck of dogs 20 hours after pancreatectomy. This technique has been used by Gayet and Guillaumie (1927, 1928), by Gayet (1933), and by Houssay, Lewis, and Foglia (1928, 1929).

The extirpation of the pancreas was performed aseptically in dogs anesthetized with

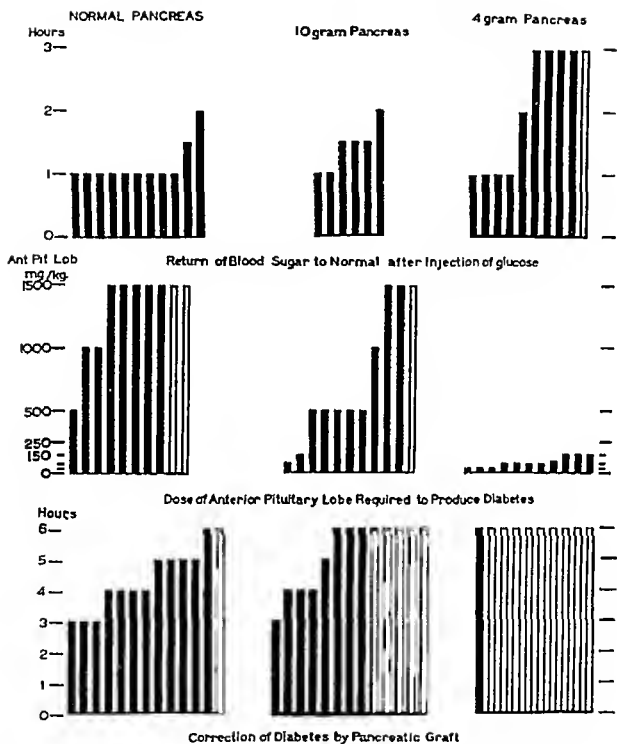


FIG 1 Each column corresponds to one experiment in a different animal. First row. In black, time in hours for the return of blood sugar to 120 per cent or lower after the intravenous injection of 1 gm of glucose per kilo. In white, glycemia not falling to 120 per cent. Second row. In black, daily intraperitoneal dose of alkaline extract of fresh bovine anterior pituitary gland per kilo per day for 4 days which raised the blood sugar to 150 per cent or more. In white, this degree of hyperglycemia was not produced. Third row. Pancreatic graft in neck. In black, time in hours necessary for the fall of diabetogenic hyperglycemia to 120 per cent or less. In white, cases in which hyperglycemia did not fall to this level in 6 hours.

ether In order to leave only 4 gm of the pancreas, the free end or duodenal tail was resected, and of this 4 gm were weighed and the size of this sample served as a gauge for the amount of pancreas to be left intact A ligature was placed in the side of the gland adhering to the duodenal wall and the splenic end was resected Starting near the pylorus, the pancreas was extirpated until there remained only a mass of tissue around the main pancreatic duct of Santorini of a size equal to the 4 gm portion previously weighed

To leave *in situ* about 10 gm of pancreas, the free duodenal and the splenic end were resected and the pancreatic tissue beside the duodenum was left untouched The weight of the remaining portion varied from 8.5 to 11.5 gm as calculated by subtracting the weight of the amount resected from the average weight of the pancreas of several dogs of similar body weight Alizón Garcia and Lewis (1933) have

TABLE V

Rise of Glycemia Caused by Action of Extract of Fresh Anterior Lobe of Bovine Hypophysis by Intraperitoneal Route during 4 Successive Days in 2 Dogs with Pancreas Reduced to 10 Gm

Anterior lobe daily	Dog 2-6					Dog 5-4				
	Blood sugar					Blood sugar				
	Days after injection					Days after injection				
	Before	1	2	3	4	Before	1	2	3	4
mg per kg	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
40	84	80	82	78	101	97	90	84	105	89
80	80	114	127	123	144	78	94	91	96	83
150	74	105	102	128	151	70	83	78	91	89
250	111	114	123	181	177	62	76	88	99	79
500	—	—	—	—	—	74	84	82	94	103
1000	—	—	—	—	—	69	87	83	93	141
1500	—	—	—	—	—	78	88	107	122	114
2000	—	—	—	—	—	82	102	93	112	211
Sensitive to 150 mg per kg daily						Sensitive to 2000 mg per kg daily				

made determinations on 193 dogs to establish the ratio between body weight and the weight of the pancreas In a high percentage of dogs of 7.5 to 10 kilos the pancreas weighed 16 to 20 gm

Glycemia before and after Meals—The glycemia was determined in the morning after a fasting period of 18 hours, the animals being accustomed to the laboratory and quiet In these cases the mean fasting blood sugar in dogs with 4 gm of pancreas was 89 mg per cent, in those with 10 gm it was 91 mg per cent, and in normal dogs 90 mg per cent In some dogs the blood sugar was determined before, during, and after a meal consisting of 35 gm of raw beef per kilo of body weight The variations observed in the glyceimic curve were substantially identical in the three groups of animals

Glycemic Curve after Injection of Glucose—Although the three groups of animals had glycemia within the normal levels, the intravenous injection of 1 gm of glucose

per kilo of body weight produced a condition of strain on the pancreas and revealed that some of the animals with reduced pancreas had a more prolonged hyperglycemia than the normal animals. The glycemia returned to a level of 120 mg per cent in an hour in 9 out of 11 normal dogs, in 1½ to 2 hours in 4 out of 6 dogs with 10 gm of pancreas and in 3 or more hours in 5 out of 10 dogs with 4 gm of pancreas (Tables I to IV and Fig 1).

Diabetogenic Doses of Extract of Anterior Hypophysis—A 20 per cent saline or alkaline extract of anterior lobe of fresh raw hypophysis was injected intraperitoneally. The daily dose was administered in two injections, one in the morning and the other in the afternoon for 4 successive days. When the glycemia determined in the morning and 18 to 20 hours after the last ingestion of food reached a level of 150 mg per cent or higher the result was considered positive. The factors that may alter the results are the potency of the extract, the diet, and the sensitivity of the animal. The glycemia does not increase if the animal does not eat. When the daily dose was ineffective a larger dose was injected the following week and if this dose was also ineffective a still larger dose was tried until that dose was found which produced the required degree of hyperglycemia (150 mg per cent) (Table V).

RESULTS

In Table VI and Fig 1, the results obtained are summarized. 70 per cent of normal dogs gave positive results with 1500 mg of fresh anterior lobe of the hypophysis per kilo per day, the maximum glycemia observed was of 3.22 per cent, the average 1.78 per cent. With 1000 mg per kilo per day there were 53 per cent positive results and with 500 mg per kilo per day only 14 per cent were positive. Seven out of 12 animals with about 10 gm of residual pancreas were more sensitive to the extract of the hypophysis, there was hyperglycemia higher than 1.5 per cent in 5 with daily doses of 500 mg per kilo, in one with 150 mg, and in one with 80 mg. In the last 2 animals, the pancreas showed a marked insufficiency in its capacity to resist the action of pituitary extract. In 5 animals, however, the resistance was similar to that of control animals with a normal pancreas.

In dogs with 4 gm of pancreas a great sensitivity to the diabetogenic action of the hypophysis was observed. Positive results were observed in all the animals with doses of 40 to 150 mg per kilo per day, in other words with doses 10 to 25 times smaller than those that produced positive results in about 50 per cent of the control animals in 4 days.

In addition to the animals mentioned in Table VI, there were 9 hypophysectomized dogs with 4 gm of pancreas. For these the dose of anterior hypophysis extract required to produce glycemic levels higher than 1.5 per cent (frequently higher than 2 per cent and in a few instances reaching 3 per cent) was 20 to 40 mg per day and kilo.

In the great majority of the injected animals examination of the liver revealed a fatty appearance regardless of the status of glycemia. A resistance to the action of insulin induced by extracts of anterior hypophysis first noted in 1933

in dogs in this Institute has been repeatedly observed since that time. This action has been found in animals with or without hyperglycemia and in dogs with reduced pancreas.

The sensitivity of the dogs to the action of the extract of hypophysis was fairly constant but in a few cases it did increase or diminish slowly. For example dog 7-5 with 4 gm of pancreas showed the same sensitivity to 80 mg of extract per kilo of body weight in determinations made on August 19 and October 21, and dog 7-3 gave a positive result with 250 mg of extract on September 2 and also a positive result with 80 mg on October 21.

TABLE VI

Daily Dose of Fresh Anterior Lobe of Bovine Hypophysis in Mg per Kilo That Caused Increase of Glycemia to 150 Mg Per Cent within 4 Days

Control dogs	Mg per kg of weight								
	40	80	100	150	250	500	1000	1500	2000
Total No. of dogs						21	38	74	
No. with glycemia exceeding 150 mg per cent						3	20	54	
Percentage of animals with glycemia exceeding 150 mg per cent						14	53	70	
Dogs with 8 to 10 gm pancreas with glycemia level higher than 150 mg per cent		1		1		5	1	2	1*
Dogs with 4 gm pancreas with glycemia level higher than 150 mg per cent	3	4	1	3					

* The glycemia level did not reach 150 mg per cent even with doses of 2500 mg of anterior lobe

Secretion of Insulin —

The duodenum-pancreas was grafted in the neck of chloralosed diabetic dogs, pancreatectomized 24 hours before. By means of Payr's cannulas, the carotid artery of the receptor was anastomosed to the celiac artery of the duodenum-pancreas and the jugular vein to the portal vein of the graft. The temperature of the irrigated graft was maintained at about 38°C by a thermostatic control. All results obtained from experiments in which there was a faulty circulation through the graft or visible abnormalities of the pancreas or cyanosis or death of the receptor dog, were discarded. With the pancreas of about 10 gm the graft was perfused for 6 to 8 hours and with that of 4 gm the graft was perfused for 8 to 10 hours.

The grafted pancreas regulates the glycemia, replacing the function of the pancreas *in situ*. The glycemia is maintained within the normal levels and insulin is secreted according to the humoral stimulation and the glycaemic level. It prevents or corrects the diabetic hyperglycemia. For example, in a dog

pancreatectomized 20 hours before, the grafting of a duodenum pancreas produces a rapid decrease of the glycemia, reaching the normal level in 3 to 5 hours

TABLE VII

Graft of Pancreas to Carotid and Jugular of Dogs Made Diabetic by Pancreatectomy 24 Hours Previously Number of Cases in Which the Glycemia of the Receptor Returned to a Level of 120 Mg Per Cent or Lower in a Given Number of Hours

Pancreas grafted	Decrease in hrs				No decrease in 6 or more hrs	Summary		
	3	4	5	6		Total No of animals	Decrease in 3-5 hrs	Decrease in 6 or more hrs
Total and normal	3	4	4	1	1	13	11	2
Reduced to 8-10 gm	1	3	1	3	5	13	5	8
Reduced to 4 gm	—	—	—	1	9*	10	0	10

* Two dogs had decrease in 7 hours remaining 7 had no decrease during the 8 to 10 hours under observation

TABLE VIII

Graft of Duodeno-Pancreas of Normal Dogs to the Neck of Dogs Pancreatectomized 24 Hours before

Dog No	Weight receptor dog kg	Blood sugar at end of given hrs											Weight of donor dog kg
		Before	½	1	1½	2	3	4	5	6	7	8	
		mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	
1	—	294	268	246	210	184	119	98	95	104	107	118	7 0
2	—	242	213	210	202	188	121	105	82	77	78	71	8 0
3	—	202	185	165	156	124	119	100	111	101	—	—	7 2
4	13 5	257	265	257	257	225	162	126	119	100	—	—	14 0
5	13 0	352	296	277	239	226	188	127	105	100	—	—	12 0
6	12 5	280	221	260	217	—	187	160	138	108	—	—	11 3
7	15 5	239	220	205	198	211	205	185	168	159	—	—	14 0
8	12 0	227	197	179	172	136	—	124	97	97	—	—	16 5
9	12 0	258	224	222	201	192	142	129	98	89	—	—	12 0
1-0	16 0	213	180	176	187	173	149	109	100	87	—	—	12 5
1 1	13 0	231	216	199	155	126	132	98	95	91	—	—	11 5
1 2	11 0	202	209	195	166	135	109	93	90	82	—	—	10 0
1 3	9 0	217	227	239	227	198	125	118	113	119	—	—	12 3

without surpassing it (Tables VII and VIII) For further information see Houssay (1937)

The average speed in the fall of the glycemia of the receptor pancreatectomized dog gives an indication of the amount of insulin secreted in the time unit Gayet (1928, 1933) has observed that the fall of the glycemia is more

TABLE IX

Graft of Duodeno-Pancreas of Dogs with Reduced Pancreas by Pancreatectomy to 10 Gm in the Neck of Dogs Pancreatectomized 20 Hours before

Donor					Receptor												
Dog No	Weight	Days after operation	Glycemia	Weight of pancreas	Dog No	Weight	Blood sugar after given hrs										
							Be fore	½	1	1½	2	3	4	5	6	7	8
							mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
—	6 1	58	178	—	1	12 5	187	—	170	140	145	104	90	83	74		
5	9 0	26	100	12 9	2	14 5	271	236	236	236	162	130	116	112	—		
8	7 0	20	100	10 0	3	12 0	194	176	189	172	180	180	134	134	91		
9	10 0	18	94	14 7	4	17 0	458	462	484	466	458	440	440	378	376		
1-0	11 0	18	96	17 0	5	16 0	298	306	256	256	222	147	131	124	124		
1-7	10 5	20	92	12 5	6	17 0	247	245	235	207	187	167	138	122	99		
1-1	10 7	35	88	14 0	7	20 0	220	223	207	217	189	175	140	121	102		
2-0	7 0	20	102	—	8	17 0	294	266	244	214	208	164	120	101	84		
1-8	8 5	84	103	16 4	1-0	9 5	278	254	235	201	197	141	106	85	80	78	80
2-3	8 3	74	94	8 2	1-2	13 0	283	265	257	222	207	176	136	110	104	91	91
5-4	8 4	55	88	9 4	1-3	14 0	266	244	235	231	218	201	147	134	129	136	129
1-9	8 5	111	98	15 0	1-4	13 5	358	340	337	326	310	247	239	207	207	198	194
1-6	11 0	116	90	—	1-1	11 0	274	241	220	208	214	235	250	241	226	—	—

TABLE X

Graft of Duodeno-Pancreas of Dogs with Reduced Pancreas by Subtotal Resection to 4 Gm in the Neck of Dogs Pancreatectomized 20 Hours before

Donor					Receptor														
Dog No	Weight	Days post operative	Glycemia	Weight of pancreas	Dog No	Weight	Blood sugar after given hrs												
							Be fore	½	1	1½	2	3	4	5	6	7	8	9	10
							mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
5-2	8 0	53	100	7 7	1	23 0	193	179	200	137	130	130	134	123	112	114			
6-1	7 0	32	116	7 7	2	10 0	264	258	256	266	248	235	202	204	188	182	166		
5-0	8 0	58	112	8 0	3	14 0	272	242	227	220	229	227	223	191	166	176	171		
5-6	13 0	17	81	12 0	4	13 0	278	252	262	242	212	177	155	136	126	114	95		
6-4	4 5	31	138	5 2	5	13 0	287	275	247	238	236	224	192	169	148	166	148	148	
6-6	7 5	30	93	—	6	23 0	293	245	225	213	211	215	211	185	178	161	165	156	
7-5	10 0	54	96	—	7	22 0	310	261	205	195	196	205	169	153	147	152	154	150	
7-0	10 0	26	94	8 3	8	9 5	281	289	257	245	240	291	167	172	224	194	172	180	
6-2	9 0	59	96	—	9	18 0	148	146	144	142	144	146	149	144	149	144	132	140	
7-1	5 5	26	102	7 0	1-0	13 5	254	252	256	256	244	206	200	160	133	119	102	95	

rapid when the grafted pancreas comes from a heavier dog than the receptor and is slower when the ratio is reversed. A graft of reduced pancreas (3.58 to 9.8 gm) in the neck of pancreatectomized dogs was observed to produce a less rapid return to normal glycemia. Reduction of the pancreas for graft was made immediately before transplant.

In our experiments, we compared the secretion of insulin of the whole pancreas and of the pancreas reduced to 10 and to 4 gm by surgical operation carried out weeks or months before (Tables IX and X).

Grafting of the pancreas reduced the normal level of the glycemia in diabetic dogs within 5 hours in 11 out of 13 when the total gland was grafted, in 5 out of 13 when the graft had 10 gm of pancreas, and in none when the graft had only 4 gm of pancreas. The fall to the normal glycemia was not reached in a period of 5 hours in 2 out of 13 when the total pancreas was grafted, in 8 out of 13 with 10 gm of pancreas, and in 10 with 4 gm (Tables VII to X). Therefore, normal secretion of insulin occurred in only 5 of the 13 with a pancreas of 10 gm (Tables VII and IX), and in none with a pancreas of 4 gm (Tables VII and X).

In the diabetic dogs in which glycemia did not fall to the limit of 120 mg per cent or below there was a very slow but gradual decrease in the blood sugar which showed that the graft of reduced pancreas irrigated with diabetic blood secreted insulin although in smaller amounts than the total pancreas did. It is possible that even the reduced pancreas might have been able to reduce the degree of intensity of the diabetes if the experiment had been carried on for a longer period of time.

DISCUSSION

The pancreas reduced to 10 or 4 gm is capable of maintaining glycemia within normal levels in basic conditions or after a meal of meat, but under conditions of strain it is usually unable to react with the same speed and efficiency as the total pancreas does.

Thus in dogs with a pancreas reduced to 10 gm, which represents a half of the normal weight, there was in several cases a slight prolongation of the hyperglycemic curve produced by intravenous injection of 1 gm of glucose per kilo. However, in some animals with pancreas reduced to 10 or 4 gm, the time of return to normal of the blood sugar was the same as in the normal controls. In the dogs with a pancreas reduced to 4 gm ($\frac{1}{4}$ to $\frac{1}{5}$ of the normal weight) there was in half of the animals a longer prolongation of the curve.

The diabetogenic action of anterior lobe of fresh bovine hypophysis was observed in more than 50 per cent of the normal dogs injected intraperitoneally with a daily dose of 1000 mg per kilo during a period of 4 days, with a daily dose of 500 mg per kilo in dogs with 10 gm of pancreatic tissue, and with a daily dose of 40 to 150 mg in the dogs having only 4 gm of pancreas.

Partial pancreatectomy does not reduce the concentration of insulin per gram in the pancreatic tissue that is left, provided the extirpation is not large enough to produce diabetes, in which case there is a decrease in the concentration of insulin (Haist and Best, 1940)

By grafting the pancreas in the neck of a pancreatectomized dog it can be demonstrated by the stimulation produced by diabetic hyperglycemia that the pancreas reduced to 10 gm secretes less insulin than the total pancreas and that of 4 gm still less

The functional capacity of the reduced pancreas does not always depend on the weight of the pancreatic tissue left, for in some cases the pancreas reduced to 10 gm had a normal functional capacity whereas in a few other cases the functional capacity was reduced to that of the 4 gm pancreas

The function of reduced pancreas in some animals changed progressively, increasing or decreasing according to the changes in the islands of Langerhans (Porto)

CONCLUSIONS

The pancreas reduced to 4 or 10 gm weeks or months previously by partial resection, is able to maintain a normal glycemic level in dogs of about 10 kilos in good condition. When the pancreas is reduced to 4 gm the capacity for secreting insulin under certain conditions of strain is diminished whereas a pancreas reduced to 10 gm may have a normal or decreased capacity. This decreased functional capacity is shown (1) by a longer hyperglycemic curve after the intravenous injection of 1 gm of glucose per kilo, (2) by the requirement of smaller doses of extract of anterior hypophysis to produce diabetes, and (3) by the longer time required to correct the diabetic hyperglycemia if reduced pancreas is grafted in the neck of pancreatectomized animals. The time to recover is in inverse ratio to the weight of the transplanted pancreatic tissue

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in the assay of complementary activity. The methods that have been employed in the chemical fractionation, purification, and characterization of the proteins are those generally in use in the Department of Physical Chemistry at the Harvard Medical School (19-23) where during a sojourn of one of the authors (L. P.), the work described in this communication was carried out.

Material—Because of its high content of hemolytic complement, guinea pig serum was employed in all the purifications described below. The blood of healthy adult male guinea pigs was pooled and allowed to clot in the refrigerator at 3-4°C. After 6 hours, the clear serum was separated, by centrifugation, and immediately chilled to 1°C. Precipitation of the serum proteins was started within 1 hour.

Immunological Methods—The methods for preparing the mid-piece, end-piece, ammonia-treated serum, and zymun-treated serum employed in the immunological tests have been previously described (10, 24). Methods for the accurate titration of complement as well as the manner of reactivation have also been described in detail elsewhere (18, 10). In the following experiments heated mid-piece (heated at 58°C for 30 minutes) was the source of 3rd component.

Electrophoretic and Ultracentrifugal Methods—The electrophoretic experiments on the serum fractions were carried out in a Tiselius apparatus equipped with a schlieren optical system of a modified Philpot type (25). Electrophoretic mobilities were measured for 1 per cent protein solutions at about 4° in phosphate buffer of 0.20 ionic strength and pH 7.7, and have been expressed as mobilities at 0° (since the conductivity of the buffer was measured at 0°). Photographs were made at hourly intervals over a period of 2½ to 3 hours. The centrifugal studies were carried out in an air-driven ultracentrifuge of the Bauer-Pickels type (26), equipped with a schlieren optical system as was used in the electrophoresis apparatus. The centrifuge was driven at a speed of 54,000 R.P.M., and photographs were made at 10 or 20 minute intervals. In nearly all cases sedimentation constants were measured for 1 per cent protein solutions in potassium chloride of 0.20 ionic strength, and the sedimentation constants, $s_{20}^{1\%}$, have been corrected to the density and viscosity of water at 20°, but not to zero protein concentration.

Chemical Fractionation of the Components of Complement

Ecker, Pillemer, Jones, and Seifter (2) have shown that the addition of 14 volumes of 2.4 M $(\text{NH}_4)_2\text{SO}_4$ to 1 volume of guinea pig serum results in the precipitation of about 35 per cent of the total serum protein containing about 90 per cent of the complementary activity of the serum. However, attempts to separate this material into the various components of complement were futile. It became evident that although the 1:15 dilution of serum with the desired $(\text{NH}_4)_2\text{SO}_4$ concentration was sufficient to separate the globulin from serum without much loss of complementary activity, the same method was unsatisfactory for further purification of the complement components.

The method adopted for the purification of the complement components was that described by Cohn, McMeekin, Oncley, Newell, and Hughes (21, 23).

The serum proteins were maintained at a high concentration throughout and reagents added by slow diffusion through a rotating cellophane membrane. All procedures were carried out at 1°C and conditions of pH and ionic strength were strictly controlled.

Four preparations were carried to completion.

Preparation I—In this preliminary preparation 90 cc of serum were employed. The results indicated (1) that less than 25 per cent of the total serum proteins were involved in complementary activity (2) that serum proteins insoluble in 1.39 M $(\text{NH}_4)_2\text{SO}_4$ contained all of the mid piece (3) that the material soluble in 1.39 M but insoluble in 2.0 M $(\text{NH}_4)_2\text{SO}_4$ was devoid of all components of complement with the exception of 3rd component and (4) that the material soluble in 2.0 M but insoluble in 2.5 M $(\text{NH}_4)_2\text{SO}_4$ contained both the end piece and 4th component. Because of the small amount of material in each fraction further purification was not feasible and no satisfactory analytical data were obtained.

Preparation II—225 cc of guinea pig serum were diluted with 180 cc of 0.9 per cent NaCl, the solution was chilled to 1°C and then carefully mixed with 225 cc of 3 M $(\text{NH}_4)_2\text{SO}_4$ and 45 cc of N/10 H_2SO_4 . All of the reagents were brought to 1°C before mixing and this temperature was maintained during the preparation of the various serum proteins. The final protein concentration was 2.3 per cent and the pH was maintained near 6.0, since 4th component is destroyed by free amino groups (8, 10), whereas as Pillemer, Seifter, and Ecker have shown (12), this component as well as end piece is unstable at acid reactions.

The desired amount of 3 M $(\text{NH}_4)_2\text{SO}_4$ for fractionation of the protein fractions was introduced into a cellophane membrane and allowed to diffuse slowly with rotation into the protein solution until the desired equilibrium was attained. This was accomplished in every instance in not more than 18 hours. The first equilibration was carried to 1.39 M $(\text{NH}_4)_2\text{SO}_4$, the proteins insoluble at this salt concentration removed by centrifugation in a refrigerated centrifuge and the clear supernatant decanted. The 1.39 M $(\text{NH}_4)_2\text{SO}_4$ precipitate was then washed three times with 1.39 M $(\text{NH}_4)_2\text{SO}_4$ to avoid occlusions and the washings added to the solution to maintain the original volume.

The precipitate was then dissolved in the smallest possible volume of distilled water or buffer transferred to a cellophane membrane, and dialyzed with rotation against large volumes and repeated changes of distilled water or buffer (see section on purification of mid piece). After 24 hours the dialyzing medium was usually ammonia free and the dialyzed material transferred to ice-cold tubes, centrifuged, the supernatants decanted and the precipitate washed three times with the dialyzing fluid as before. The washings were added to the supernatant solution. The precipitate was then dissolved in a minimum amount of 0.9 per cent NaCl, and a sufficient amount of NaCl added to the supernatants to render them isotonic. In a similar manner precipitates were collected successively at 1.68 M, 2.0 M, 2.2 M, 2.5 M, and 3.4 M $(\text{NH}_4)_2\text{SO}_4$. The fractions were immediately tested for their complementary activity (Table I), and subsequently characterized electrophoretically and in the ultracentrifuge (Table II). The euglobulin part of the precipitate at 1.39 M $(\text{NH}_4)_2\text{SO}_4$ contained all of the mid piece and a small amount of 3rd component. This fraction, while appearing

homogeneous in the ultracentrifuge, contained at least three proteins as judged by electrophoretic analysis. The serum proteins soluble at 1.39 M but insoluble at 2.0 M $(\text{NH}_4)_2\text{SO}_4$ contained no active complement components with the exception of a small amount of 3rd component² in the euglobulins, although this material was composed of about 20 per cent of the total serum proteins and approximately 80 per cent of the serum globulins. It was noted (1) that the water-soluble material precipitated be-

TABLE I

Distribution of Complement Substances in the Ammonium Sulfate Fractionation of the Serum Proteins of the Guinea Pig

Ammonium sulfate concentration <i>mole/liter</i>	Solubility in water*	Protein precipitated <i>per cent of total</i>	Content of complement substances in each serum fraction			
			Mid piece <i>per cent</i>	End piece <i>per cent</i>	3rd component <i>per cent</i>	4th component <i>per cent</i>
1.39	Soluble	7	0	0	15	10
	Insoluble	4.6	100	0	30	0
1.60	Soluble	5	0	0	0	0
	Insoluble	4.4	0	0	30	0
2.00	Soluble	5.5	0	0	0	0
	Insoluble	2.1	0	0	20	0
2.20	Soluble	5.3	0	60	0	50
	Insoluble	0.2	0	20	0	30
2.50	Soluble	50	0	0	0	0
	Insoluble	Trace	0	0	10	0
3.40	Soluble	12	0	0	0	0
Total of proteins isolated		96.1	100	80	105	90

* Insolubility here designates the fraction precipitated at each $(\text{NH}_4)_2\text{SO}_4$ concentration which separates upon dialysis against cold distilled water, except the 1.39 M euglobulin fraction which was separated by dialysis against phosphate buffers of pH 5.2-5.3 and ionic strength 0.02.

tween 2.0 M and 2.2 M $(\text{NH}_4)_2\text{SO}_4$, which contained 5.3 per cent of the total serum proteins, and which behaved electrophoretically as though it were albumin, contained

² The 3rd component of complement was found in small quantities in nearly every fraction of serum. Evidence indicating that it may be a phospholipid or closely associated with phospholipids has elsewhere been presented (24). Recently Pillemer and Ecker (11) have isolated an insoluble constituent from fresh yeast which specifically adsorbs all of the 3rd component from serum. Attempts are now in progress by them to isolate and purify this complement component.

TABLE II

Distribution of Proteins in the Ammonium Sulfate Fractionation of Guinea Pig Serum as Estimated by Electrophoretic and Ultracentrifugal Analyses

Ammonium sulfate concentration	Solubility in water	Mobilities of serum proteins in phosphate buffer solutions of 0.2 ionic strength at pH 7.7 $\mu_0^{17} \times 10^6$					
		Albumin	Globulins				
		4.7	3.8	2.9	2.5	1.8	1.0
Amount of protein with each mobility in serum and in the fractions							
mole/liter		per cent	per cent	per cent	per cent	per cent	per cent
Total serum		70	10	20			
1.39	Soluble		0.1			4.9	2.0
	Insoluble*		0.1	4.3	0.2		
1.60	Soluble		0.1			2.5	2.4
2.00	Soluble		1.9	0.2	1.7		1.7
2.20	Soluble†	5.2	0.1				
2.50	Soluble	4.8	1.8	0.2			
Sedimentation constants of serum proteins in 1 per cent solution $s_{20}^{17} \times 10^{13}$							
		17	6.4	4.3	3.1		
Amount of protein with each sedimentation constant in serum and in the fraction							
Total serum		per cent	per cent	per cent	per cent		
		5	25	70			
1.39	Soluble		7.0				
	Insoluble‡		4.6				
1.60	Soluble		5.0				
2.00	Soluble		4.1			1.4	
2.20	Soluble§			5.3			
2.50	Soluble	1		4.9			
3.40	Soluble			12			

* In the final preparation of purified mid piece from this fraction of serum 0.6 per cent of the total protein was separated and an electrophoretic mobility of 2.9×10^{-5} was observed with not more than 0.01 per cent of the total serum protein with a lower mobility.

† In the final preparation of purified end piece and 4th component from this fraction of serum only 0.2 per cent of the total protein was separated and an electrophoretic mobility of 4.2×10^{-5} was observed with not more than 0.004 per cent of the total serum protein of mobility slightly higher.

‡ In the final preparation of purified mid piece from this fraction of serum 0.6 per cent of the total protein was separated and a sedimentation constant of 6.4×10^{-13} was observed upon ultracentrifugal analysis.

§ In the final preparation of purified end piece and 4th component from this fraction of serum 0.2 per cent of the total protein was separated and sedimentation constants of 17.63 and 3.8×10^{-13} in amounts of the total serum protein of about 0.01, 0.07 and 0.12 per

60 per cent and 50 per cent end-piece and 4th component respectively, (2) that a trace of euglobulin which settled out of the 2.2 M fraction on dialysis against distilled water contained respectively 20 per cent and 30 per cent of these components. This was the first indication that they might be euglobulins in nature, since previously it had been thought that these were either pseudoglobulins (10) or albumins (8).

The various serum fractions were frozen, dried, and sealed *in vacuo* (27) without much loss of solubility or content of complement components (3rd component being an exception discussed subsequently in the section dealing with mid-piece). This permitted the accumulation of larger amounts of fractionated serum for the characterizations of the complement substances.

Preparations III and IV—460 cc of serum from 79 guinea pigs and 610 cc of serum from 97 guinea pigs were processed identically as in Preparation II. The pseudoglobulins and albumins were frozen and dried in a salt-free condition, while the euglobulins were dried in a minimum amount of 0.9 per cent NaCl, since it had been determined in Preparation II that better re-solution of the euglobulins occurred when they were treated in this manner. The dried material was stored at 3–4°C in vacuum-sealed bottles.

Upon completion of the two preparations, the sealed bottles of identical fractions were opened, tested for complementary activity, and combined. The serum fractions containing active complement components were then maintained in a frozen state in a –15°C bath, as it was felt that such treatment would be less drastic than repeated freezing and dehydration. Characterization of the various serum protein fractions indicated that almost identical fractionations were obtained in Preparations II, III, and IV.

Purification and Characterization of the Mid-Piece Component of Complement

The euglobulins insoluble at 1.39 M $(\text{NH}_4)_2\text{SO}_4$ contained all of the mid-piece. The further purification and fractionation of the crude euglobulin precipitate separating at this ammonium sulfate concentration were therefore undertaken.

Earlier investigators (24, 28) believed that mid-piece was very unstable and lost activity in a few hours time, becoming anticomplementary. Browning and Mackie (4) and Parsons (5) failed to observe any complementary activity or components in the precipitates they obtained at a third saturation of $(\text{NH}_4)_2\text{SO}_4$, and suggested that this serum fraction was anticomplementary. In the present studies also, activity was not observed in the crude, initial preparations. Stability studies of the mid-piece euglobulin have shown, however, that this component of complement, while very unstable at neutral reactions, is stable at a slightly acid reaction (12). Therefore, the practice has been to maintain the pH of the purified protein between 5.4 and 5.8, only neutralizing to pH 7 before testing for complementary function. No mid-piece activity, furthermore, was observed at protein concentrations exceeding 0.02 per cent, while full activity was always observed at protein concentrations between 0.002 and 0.02 per cent.

The crude 1.39 M $(\text{NH}_4)_2\text{SO}_4$ precipitate contained quite large amounts of fat in suspension, giving a milky appearance. The yield from 1070 cc of serum or 65 gm of protein was 2.7 gm, or 4.1 per cent of the total serum proteins. Eder, Pillemer,

and Grabill (29) showed that extraction of dried complement with fat solvents resulted in increased complementary activity of serum, and that the addition of such extracted fats to normal guinea pig serum had an anticomplementary effect. Therefore, the globulins precipitated were dissolved in a small amount of 0.9 per cent NaCl neutralized to 5.8 with 0.01 N NaOH and centrifuged in the cold for 2 hours at 2750 R P M. Nearly all of the suspended fat collected at the surface and was skimmed off. The

TABLE III

Further Fractionation in Phosphate Buffers Varying in pH and Ionic Strength of Precipitate from Guinea Pig Serum at 1.39 M $(\text{NH}_4)_2\text{SO}_4$ at 1 C

Ionic strength of phosphate buffer	pH	Protein insoluble	Solubility in phosphate buffer	Content of complement substances			
				Mid piece	End-piece	3rd component	4th component
$\Gamma/2$		per cent		per cent	per cent	per cent	per cent
0.00*	6.4	75	Soluble	0	0	0	Trace
			Insoluble	0	0	0	0
0.10	6.4	30	Soluble	0	0	0	15
			Insoluble	0	0	0	0
0.10	7.0	30	Soluble	0	0	0	15
			Insoluble	0	0	0	0
0.01	5.2	75	Soluble	0	0	0	10
			Insoluble	100	0	60	0
0.02	5.2	60	Soluble	0	0	45	15
			Insoluble	100	0	15	0
0.05	5.2	50	Soluble	40	0	45	15
			Insoluble	60	0	15	0
0.075	5.2	50	Soluble	40	0	45	15
			Insoluble	60	0	15	0

* The dialysis in this case was against distilled water.

protein solution filtered through hardened filter paper was almost completely clear and exhibited full complementary activity.

Judged both electrophoretically and immunologically the euglobulin precipitated by 1.39 M $(\text{NH}_4)_2\text{SO}_4$ was far from being homogeneous. Since it was known that the mid piece is a euglobulin with a solubility minimum in the region of pH 5.2 (15), the following experiments were carried out to determine the conditions for its specific and quantitative precipitation.

The 1.39 M precipitate was dialyzed against phosphate buffers of ionic strengths ranging from 0.01 to 0.10 and pH from 5.2 to 7.0. Dialysis against distilled water or phosphate buffer at a pH of 6.4 resulted in a complete loss of all activity (except that of the 4th component) while dialysis against phosphate buffer at ionic strengths

of 0.05 to 0.075 and a pH of 5.2 resulted in no loss of mid-piece activity and showed a varying distribution between precipitates and solution. The results of this experiment (Table III) demonstrate that whereas the active euglobulin was quantitatively precipitated at an ionic strength of 0.01 or 0.02 at pH 5.2, a fair proportion of other euglobulins as well as pseudoglobulin remained in solution, thus leading to almost substantial purification. At an ionic strength of 0.02 at a pH of 5.2, 4th component appeared to remain in solution with most of the 3rd component originally present in the 1.39 M precipitate.

The euglobulin separating at an ionic strength of 0.02 at pH 5.2 from the protein fraction precipitated by 1.39 M $(\text{NH}_4)_2\text{SO}_4$ at 1°C , although containing all of the mid-piece activity, was still not homogeneous electrophoretically. At least four proteins were present, of which one, constituting 85 per cent of the material, had an electrophoretic mobility of about 2.9×10^{-5} , the remaining three having mobilities of 1.0, 2.5 and 3.8×10^{-5} respectively, when measured in phosphate buffers of ionic strength 0.02 at pH 7.7.

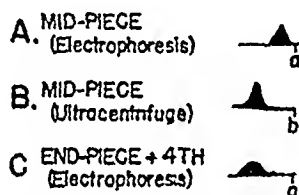


FIG. 1. Schlieren patterns of purified components of complement. Electrophoretic experiments in phosphate buffer, pH = 7.7, $\Gamma/2 = 0.20$, ultracentrifuge experiments in 0.20 M potassium chloride. *a*, location of initial boundary (diagram is of descending boundary), *b*, location of meniscus.

The euglobulin dissolved at a concentration of 10 per cent in 0.9 per cent NaCl was therefore reprecipitated by again diffusing $(\text{NH}_4)_2\text{SO}_4$ through a cellophane membrane until the equilibrium concentration was 1.22 M. The resulting precipitate, redissolved and again dialyzed against a phosphate buffer of ionic strength 0.02 at pH 5.2, yielded reprecipitated euglobulins retaining full mid-piece activity.

This euglobulin separated by the above procedures was considered sufficiently homogeneous to be studied electrophoretically and in the ultracentrifuge. Fully 98 per cent of the precipitate had an electrophoretic mobility of 2.9×10^{-5} , and but a small amount of material migrated at a slower velocity (Fig. 1 A). A schlieren diagram of an ultracentrifugal analysis is represented in Fig. 1 B and reveals that all of the molecules were sedimenting with the same velocity, calculated to be 6.4×10^{-13} .

The final yield of this protein from 1070 cc. of serum was 400 mg., or 0.6 per cent of the total protein. Upon drying this material a small amount, which proved not to be the mid-piece, failed to redissolve in a 0.9 per cent NaCl solution. The greater stability of the mid-piece of complement under these conditions thus served to increase the final purification.

Purification and Characterization of End-Piece and 4th Component

The serum proteins soluble in 2.0 M but insoluble in 2.2 M $(\text{NH}_4)_2\text{SO}_4$ contain 80 to 85 per cent of both end piece and 4th component, and comprise approximately 5 per cent of the total serum protein. The yield from 1070 cc of serum was 3 gm. In Preparation II, a slight amount of material had precipitated during dialysis against distilled water, which contained both of these components of complement, suggesting that these proteins might also be euglobulins.

The serum proteins soluble at 2.0 M $(\text{NH}_4)_2\text{SO}_4$ were therefore adjusted carefully to a pH of 6.4 with NaOH, and 3 M $(\text{NH}_4)_2\text{SO}_4$ previously neutralized to a pH of 6.4 was allowed to diffuse through a rotating cellophane membrane into the protein solution until a molarity of 2.2 M $(\text{NH}_4)_2\text{SO}_4$ had been attained. The precipitated material was washed five times with 2.2 M $(\text{NH}_4)_2\text{SO}_4$ dissolved in a minimum amount of distilled water, and dialyzed against repeatedly changed conductivity water. Usually, after 48 hours a green, transparent extremely viscous precipitate separated, which clung tenaciously to the sides of the cellophane membrane. The precipitate was scraped from the cellophane membrane into its mother liquor with a spatula, and centrifuged. It separated as a green transparent gel much like the fraction designated P_1 by Green (19). Upon resuspension of this material in distilled water it formed a white, amorphous suspension which upon recentrifugation again separated as a green, transparent gel. The gel was washed five times with conductivity water, and finally dissolved in a minimum quantity of 0.9 per cent NaCl. This material contained all of the end piece and 4th component originally present in the 2.0 M-2.2 M precipitate. This euglobulin fraction was further purified by being twice reprecipitated by $(\text{NH}_4)_2\text{SO}_4$ and twice reprecipitated by dialysis against conductivity water.

The muco-euglobulin separated by the procedure given in detail above contained 85 per cent of both components (end piece and 4th component). Its carbohydrate content as determined by the Orcan method (30) was 10.3 per cent as contrasted with a carbohydrate content of end piece of 2.7 per cent. The final yield of this active precipitate was 120 mg or less than 0.2 per cent of the total serum protein. Its specific optical rotation was -192.5° , and it reduced Schiff's reagent (31). It contained but a very small amount of phosphorus.

Treatment of this muco-euglobulin at 50°C for 30 minutes destroyed all of its end piece activity, but did not inhibit 4th component. It appears that this protein fraction retained two functions, one by virtue of a relatively heat labile, and the other by virtue of a relatively heat stable component.

Gordon, Whitehead, and Wormall (8) have shown that the "albumin fraction" of serum contains both heat labile and heat stable components of complement. Further studies by Pillemer, Seifter, and Ecker (10, 12, 32) have revealed that these components are relatively stable in alkaline solutions, but unstable in slightly acid solutions; that the heat-stable component is inactivated

by organic compounds that react with carbonyl groups, and that calcium and lipids play little or no rôle in the activity of either of the two components. The hypothesis was offered that the two components are a carbohydrate-protein complex. The unusually high carbohydrate content of the muco-euglobulin that has now been separated and its ability to reduce Schiff's reagent is not inconsistent with this hypothesis. This serum fraction exhibited full end-piece and 4th component activity in dilutions of 0.01 per cent and showed no inhibitory action of the kind discussed for mid-piece.

TABLE IV
Properties of Purified Components of Complement

	Euglobulin	Muco-euglobulin
Complement components present	Mid-piece	End piece and 4th component
Mobility in phosphate buffer, at pH 7.7, 0.2 ionic strength	2.9×10^{-5}	4.2×10^{-5}
Sedimentation constant, $s_{20}^{1\%}$, s_w	6.4×10^{-13}	—
Protein nitrogen, per cent	16.3	14.2
Carbohydrate, per cent	2.7	10.3
Phosphorus, per cent	>0.1	>0.1
Optical rotation, $^\circ (\alpha)_{25}^D$	-28.7	-192.5
Apparent isoelectric point	5.2-5.4	6.3-6.4
Fraction of total complement activity, per cent	100	85
Fraction of total serum protein, per cent*	0.6	0.18
Heat stability of complement, activity (destroyed in 30 min at tabulated temperature), $^\circ\text{C}$	50	50,† 66‡

* The three complement components together then comprise 0.78 per cent of the total serum protein. Heidelberger (33) reported that by an entirely different technique he found that 1 cc of guinea pig serum contains from 0.15 to 0.2 mg of complement protein. However, in a personal communication to the authors, Heidelberger reports that he now finds 0.4 to 0.7 mg of complement protein to be a safer approximation. This is in good agreement with the values reported in this paper.

† For end piece activity.

‡ For 4th component activity.

Electrophoretic analysis revealed that this muco-euglobulin fraction had not more than 2 per cent of slower moving components, with 98 per cent having a mobility of 4.2×10^{-5} in phosphate buffer of 0.2 ionic strength at pH 7.7 (Fig. 1 C). This mobility is characteristic of the fastest moving globulins, termed α -globulins by Tiselius. Ultracentrifugal analysis of this fraction, because of technical difficulties, was not carried out until 24 days after its preparation. Although this material was kept at 3-5 $^\circ\text{C}$, about half of it was insoluble at the conclusion of this period of time. The part of the fraction which remained soluble revealed several boundaries upon ultracentrifugal analysis. The sedimentation constants observed, 17, 6.3, and 3.8×10^{-12} (with

amounts respectively of 5, 35, and 60 per cent), are close to those previously reported for purified α globulins from other species (21, 23). Since all of the available material was exhausted in other characterizations of this serum fraction, no repetition could be made, but as soon as more material is available, a study will be undertaken to determine if the fraction containing these components of complement represents a single component in the ultracentrifuge, several independent components, or several components in equilibrium with one another.

Meanwhile, the properties of these purified fractions containing (1) mid piece, and (2) end piece and 4th component, are contrasted in Table IV. It will be noted that the apparent isoelectric point, that is, the pH of water triturated with the precipitated euglobulins associated with end piece and 4th component actually was close to 6.3, while that of the mid piece was 5.2. The fraction with the higher mobility at pH 7.7 and precipitated by the higher concentration of salt thus had the more nearly neutral isoelectric point. Here again, more material should make possible confirmation of this unexpected result, and a more detailed study of the interactions of these components of complement with each other, and with the other components of serum.

SUMMARY

1. Methods for the separation from guinea pig serum in highly purified form of three of the components of complement are described. These substances are the so called mid piece, end piece, and 4th component.

2. Mid piece has been separated as a euglobulin, with an electrophoretic mobility of 2.9×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7, and with a sedimentation constant of 6.4×10^{-13} in potassium chloride of ionic strength 0.2.

3. End piece and 4th component were found together in a euglobulin fraction of serum which contained 10.3 per cent carbohydrate and had an electrophoretic mobility of 4.2×10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7.

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STUDIES ON AN AGGLUTINOGEN (Rh) IN HUMAN BLOOD REACTING WITH ANTI RHESUS SERA AND WITH HUMAN ISOANTIBODIES

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From some observations made with immune sera, and particularly from the evidence provided by tests with occasionally occurring normal and post transfusion human sera containing irregular agglutinins (*cf.* reviews in 1, 2) one can conclude that there exist individual properties of human blood other than those which are demonstrable by readily available reagents such as A₁, A₂, B, M, N. Doubtless numerous attempts have been made to discover additional agglutinogens by the familiar technique used for the demonstration of the factors M and N (3), that is, with immune sera prepared by the injection of human blood into rabbits, but only few results were obtained (*e.g.* 4, 5), and these were not followed up because it was difficult to produce the immune sera again. Other ways of approaching the problem were therefore desirable and it was thought that new results might be obtained by immunizing with animal instead of human blood, considering that the blood of some animals contains antigens related to agglutinogens present in individual human bloods, for instance the Forssman substance related to A in sheep cells. A result that favored this plan was the observation that certain anti *rhesus* immune sera contain agglutinins specific for the human agglutinin M (6).

Pursuing this idea, by immunizing rabbits with *rhesus* blood an immune serum was obtained with which an agglutinable factor different from A, B, M, N, or P was detected (7), and this new factor was designated as Rh to indicate that *rhesus* blood had been used for the production of the serum. The property was then found to be present in the blood of about 85 per cent of white individuals examined (7, 8).

Evidence that the property Rh is of clinical importance was obtained when one of the writers came into possession of blood samples from patients who had shown hemolytic reactions, one with fatal outcome, after receiving repeated

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transfusions of blood of the proper group (8) The serum of these patients contained anti-Rh isoagglutinins while in the blood cells the factor was lacking This showed that the agglutino-gen in question, unlike M and N, is endowed with the capacity to induce the formation of immune isoantibodies in certain human beings

Another related fact is the appearance of immune isoantibodies in pregnancy Levine and Stetson (9) had previously reported a severe accident following a transfusion of apparently compatible blood in a woman after a stillbirth and offered the explanation that the patient had been immunized by an antigen in the dead fetus, inherited from the father Furthermore, in a review of the literature by Wiener and Peters (8) it was pointed out that apparently every recorded instance in which a hemolytic reaction followed a first transfusion with blood of the proper group had occurred with intra- or postpartum patients This supported the above mentioned hypothesis that isoimmunization can result from pregnancy Further cases of transfusion reactions attributable to isoimmunization of pregnancy were reported by Levine and Katzin (10) The serum from one of the cases, in which the isoagglutinin was identified, was found to give reactions corresponding to those of Rh

In a recent paper, Levine, Katzin, and Burnham (11) described a number of cases of erythroblastosis foetalis, stillbirths, and miscarriages, which appear to be due to isoimmunization in pregnancy From their results, the authors conclude that most of the mothers developed antibodies against the Rh factor Significant additional evidence has been obtained since (23) Previously the idea of a serological explanation of erythroblastosis was advanced by Ottenberg (12) and Darrow (13)

The purposes of our own studies were to develop a practical method of testing for the presence of the Rh factor, and to investigate its heredity

EXPERIMENTAL

The rabbit immune serum described in our preliminary communication gave reactions which were definite, although considerably weaker than those obtained with common reagents for blood grouping or M,N tests Subsequent attempts at producing immune sera in rabbits by injecting *rhesus* blood gave unsatisfactory results even though feeble Rh antibodies were detectable in some of the sera Such difficulties have also been encountered in work with immune sera against other factors (P, O, etc) We then turned to the immunization of other laboratory animals and obtained sera from guinea pigs which gave reactions corresponding in specificity to those of the rabbit antibodies

For the production of the sera large guinea pigs were injected intraperitoneally with a suspension of washed red cells of *rhesus* monkeys, each animal receiving a dose corresponding to 1 cc, in later experiments to 2 cc of whole blood The injection was repeated after 5 days and 1 week later the animals were bled The sera of the

majority of animals were found to show a difference between the two sorts of blood Rb+ and Rh- and in a group of ten animals usually one or more were found that yielded sera suitable for practical diagnosis. The manner of selecting the sera is given below.

While in the case of the immune rabbit sera the reagent was prepared in the customary way by absorbing the diluted serum with negatively reacting blood it was found with several guinea pig sera that absorption with human blood resulted merely in a non specific diminution of the agglutinin content no matter whether positive or negative blood was used. This led us to test the effect of simple dilution, and indeed it was found that a distinction between positive and negative bloods could be made directly without absorbing the sera. (As an analogy, mention may be made of rabbit immune anti A sera which cannot be specifically absorbed with A cells to produce a reagent for A₁ absorption with A₂ blood serving merely to diminish the agglutinin titer.)

The method for determining suitable sera consists in making serial dilutions by halves and testing with known negative and positive blood. Those sera which show in three (or more) successive dilutions negative reactions with the former and positive ones with the latter blood are usable.

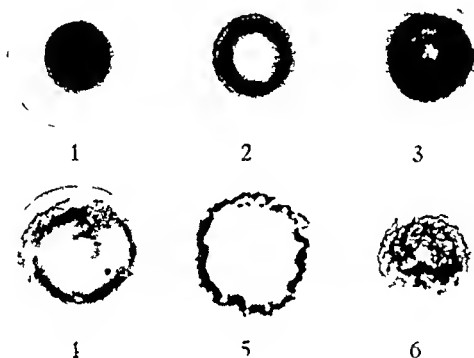
The actual tests can usually be carried out simply by selecting a dilution of the serum e.g., 1 to 10 which gives no reactions with negative but definite reactions with positive bloods. Those sera that contain appreciable amounts of anti A or anti B agglutinins having been previously absorbed with small quantities of A and B blood. Since the sera are used diluted, inactivation is mostly unnecessary. The blood to be tested should be fresh.

Another method alternative to dilution alone is to absorb the sera diluted e.g. 1:4 with a quantity of blood (using A or B cells if indicated) sufficient to remove the reaction with Rh negative blood.

Two drops (0.1 cc.) of the test fluid are then mixed with one drop of 2 per cent (in terms of blood sediment) washed blood suspension freshly prepared in a narrow tube of 7 mm. inner diameter and allowed to stand at room temperature. Readings are taken after sedimentation has occurred, usually after 30 minutes to 1 hour by direct inspection of the bases of the tubes with a hand lens. Negatively reacting bloods then show a circular deposit with a smooth edge while positive bloods have a wrinkled sediment with a serrated border or show a granular deposit (cf. Figs. 1 to 6). From these readings, as a rule, the diagnosis can readily be made. The readings are facilitated by using racks having small holes beneath the bottom of the tubes. Following the reading the tubes are shaken and the sediment examined after it forms again. A further examination is made after 2 hours again inspecting the sediment. The tubes are then gently shaken and the suspension is examined microscopically. The negative blood samples are mostly perfectly homogeneous the positive ones show various degrees of agglutination not infrequently visible to the naked eye. At times

the clumping is quite weak in spite of a distinctly positive sediment picture. Needless to say, positive and negative control bloods should be included in each test.

As already mentioned, with the great majority of specimens the distinction between positive and negative reactions is quite definite but the positive reactions vary in strength and some bloods offer difficulties because of their weak reactions. However, after sufficient practice, and by repeating the tests if necessary with fresh blood samples and several sera, only in some few instances were the reactions questionable. Marked differences in the intensity of the reactions were also observed in tests made with human anti-Rh sera. Whether the variations in strength of agglutination are due to homo- and



Magnification 1/2

FIGS 1 and 2 Negative reactions, the inner light disc in Fig 2 is due to slight convexity in the bottom of the tube

FIG 3 Faintly positive reaction

FIG 4 Weak reaction

FIGS 5 and 6 Typical positive reactions

heterozygosity or to the existence of other differences in the agglutinogen has not been determined.

In order to ascertain whether sera from various sources give corresponding reactions, comparative tests were made with series of human blood specimens. The immune rabbit serum originally obtained (7) was found to give parallel reactions with two human sera (8) from post-transfusion cases in a series of 42 bloods (29 positive and 13 negative). Additional tests have now been made to compare the reactions of different guinea pig sera with each other and with human Rh isozagglutinins. Three of the guinea pig sera were examined with a random series of 110 bloods (89 positive, 21 negative) and in no case was a discrepancy encountered. Furthermore, parallel tests were carried out with a guinea pig serum, which gave good differentiation and a human serum¹

¹ For this serum we are indebted to Dr Philip Levine and Dr P Vogel

(human serum 3) obtained from the mother of a child with erythroblastosis 159 bloods gave corresponding reactions with both sera of which 109 were positive and 50 negative. In three cases, there were definite discrepancies, one blood reacted distinctly with the human, not with the guinea pig serum, the other two were agglutinated by the guinea pig serum, not by human serum 3, though one of these was agglutinated by two other human sera (Nos 2, 4). Furthermore, three specimens gave doubtful reactions with the guinea pig serum, one reacting distinctly, one weakly, and one faintly with human serum 3. In addition to the blood mentioned above, which gave divergent reactions with the two human sera, a second such blood (not retested) was found and in a larger series of comparisons made with several human anti Rh sera, Dr Philip Levine³ observed that the reactions ran parallel in the great majority of cases, but bloods were encountered which were agglutinated differently, *e.g.* one by one serum, the other by a second serum. These observations raise the question whether there actually exist variants of the property Rh, more different than those found for agglutinogens A (14-16), M (17), and N (18), and whether human sera may contain more than one kind of anti Rh agglutinin.

Of interest in this connection is a serum, obtained from a post transfusion case (19), which differed strikingly from the other human sera in that it gave many more negative reactions. The results are summarized in Table I and show that in spite of the difference in the reactions there is a marked correlation, establishing a relationship between this serum and the other human sera. If this distribution is compared with that to be expected on the assumption that the reactions of this patient's serum would be unrelated to Rh, we obtain a value of χ^2 equal to approximately 19.9, n being 1 (cf Fisher (20)). The likelihood that this value is due to chance alone is very small.

To date a total of 448 white individuals has been examined with guinea pig sera, human sera or both.⁴ Among these, there were 379 positive and 69 negative reactions, that is, 84.6 per cent Rh+ and 15.4 per cent Rh-. The distribution in the sexes and among the blood groups and M,N types (cf Tables II to IV) did not reveal a definite correlation. Likewise in a series of 133 specimens tested for Rh and P there was no definite correlation between these two properties. The high incidence of Rh negative individuals in group B (27.5 ± 4.4 per cent) may still be accidental in view of the small size of the series.

In a series of 113 negro bloods, only 9 were found to give clearly negative reactions suggesting the possibility of a racial difference in the distribution.

The large number of negative bloods is due to selection, in order to increase the significance of the comparison.

³ Personal communication.

⁴ The children of the family material and the few instances in which the reactions with the two sorts of sera disagreed have not been included in the number.

Studies on Heredity—60 families with 237 children were tested for the presence or absence of the factor Rh in the red cells. The bloods were examined not later than 1 day following their collection. All the bloods were examined for the properties A, B, and in most cases for M, N, and the subgroups A₁ and

TABLE I

Reactions with typical human Rh sera	Reactions with human serum Wa	
	Positive	Negative
Positive	31	8
Negative	1	11

TABLE II

Distribution of the Rh Factor in the Two Sexes

	Males		Females		Totals	
	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-
Number	299	53	80	16	379	69
Per cent	84.9	15.1	83.3	16.7	84.6	15.4

TABLE III

Distribution of the Rh Factor in the Four Blood Groups

	Group O		Group A		Group B		Group AB		Totals	
	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-
Number	191	29	148	27	29	11	11	2	379	69
Per cent	86.8	13.2	84.6	15.4	72.5	27.5	84.6	15.4	84.6	15.4

TABLE IV

Distribution of the Rh Factor in the Three M, N Types

	Type M		Type N		Type MN		Totals	
	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-	Rh+	Rh-
Number	93	20	70	9	205	39	368	68
Per cent	82.3	17.7	88.6	11.4	84.0	16.0	84.4	15.6

A₂ The tests for Rh presented in Tables V and VI were made either with guinea pig immune sera, or with a post-transfusion human serum,⁵ or both. While it is realized that the use of two reagents introduces an inaccuracy, this is so small in view of the almost complete correspondence shown above between these sera that the results cannot be appreciably affected.

⁵ With the exception of 4 families, a single human serum (No. 3) was used.

TABLE V
Last of Family Material

Family Number	Parents		Children						
	Father	Mother							
1	OMNRh-	OMRh-	OMNRh- ^o	OMNRh- ^q	OMNRh- ^q	OMNRh- ^q	OMNRh- ^q	OMNRh- ^o	OJ
2	A ₂ NRh+	OMNRh+	ONRh+ ^o	A ₂ MNrh+ ^o	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^o	
3	A ₁ MNrh-	ONRh+	OMNRh+ ^q	A ₁ NRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	ONRh+ ^o	OMNRh+ ^o	
4	A ₁ MNrh+	A ₁ NRh-	A ₁ MNrh- ^q	A ₁ NRh+ ^q	A ₁ NRh- ^o	A ₁ NRh+ ^o	A ₁ NRh- ^o	A ₁ NRh- ^o	
5	A ₁ MRh+	A ₁ MRh+	OMRh+ ^o	OMRh+ ^q	OMRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
6	OMNRh+	A ₁ MRh+	OMNRh+ ^q	A ₁ MRh- ^q	OMNRh- ^o	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
7	BMRh-	OMRh-	OMRh- ^o	BMRh- ^o	BMRh- ^q	BMRh- ^q	BMRh- ^q	BMRh- ^q	
8	B ₂ MRh-	A ₂ MNrh-	A ₂ MNrh- ^q	BNRh- ^q	A ₂ MNrh- ^o	A ₂ MNrh- ^q	A ₂ MNrh- ^q	A ₂ MNrh- ^q	
9	A ₁ MRh+	OMRh+	OMRh+ ^q	OMRh+ ^q	A ₁ MRh+ ^o	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
10	OMNRh+	OMNRh+	OMRh+ ^q	OMRh+ ^o	OMNRh+ ^o	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	
11	OMNRh+	A ₁ NRh+	A ₁ NRh- ^q	A ₂ MNrh+ ^q	A ₁ MNrh+ ^q	A ₁ NRh+ ^q	A ₁ NRh+ ^q	A ₁ MNrh+ ^q	
12	OMNRh+	OMNRh+	ONRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^q	OMNRh+ ^q	
13	A ₁ MNrh-	A ₁ MNrh+	A ₁ MRh+ ^o	A ₁ MRh- ^o	OMNRh- ^q	OMNRh- ^q	OMNRh- ^q	OMNRh- ^q	
14	OMNRh+	OMNRh+	OMRh+ ^q	OMRh+ ^q	OMNRh+ ^o	OMNRh+ ^q	OMNRh+ ^q	OMRh+ ^o	OM
15	OMNRh+	A ₁ MNrh+	OMNRh+ ^q	ONRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	
16	A ₁ MNrh+	A ₁ NRh+	OMNRh+ ^q	A ₁ MNrh+ ^q	OMNRh+ ^o	A ₁ NRh+ ^o	A ₁ MNrh+ ^o	A ₁ NRh+ ^o	A ₁
17	A ₁ MNrh+	A ₁ MRh+	OMRh+ ^q	OMNRh+ ^q	OMNRh+ ^o	A ₁ MRh+ ^o	A ₁ MNrh+ ^o	A ₁ MRh+ ^o	A ₂
18	A ₁ NRh-	ONRh+	ONRh- ^o	A ₁ NRh- ^q	ONRh- ^q	ONRh- ^q	ONRh- ^q	ONRh- ^q	
	ONRh-	A ₂ MNrh-	OMNRh- ^o	ONRh- ^o	A ₂ MNrh- ^o	A ₂ NRh- ^q	A ₂ NRh- ^q	A ₂ NRh- ^q	A ₂
20	AMNRh+	A ₁ MRh+	AMNRh+ ^q	AMNRh+ ^q	AMNRh+ ^o	AMRh+ ^o	AMRh+ ^o	AMRh+ ^o	
21	OMNRh+	A ₁ MNrh+	A ₁ NRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
22	AMNRh+	BMRh+	OMRh+ ^o	AMNRh+ ^q	AMNRh+ ^q	AMRh+ ^o	AMRh+ ^o	AMRh+ ^o	
23	A ₂ MRh+	BNRh+	A ₂ BMNRh+ ^q	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	
24	OMNRh+	BMNRh-	BMNRh+ ^o	ONRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	
25	A ₁ MNrh-	A ₁ BMNRh+	BMNRh+ ^o	BMNRh+ ^o	A ₁ BMNRh+ ^o	A ₁ BMNRh+ ^o	A ₁ BMNRh+ ^o	A ₁ BMNRh+ ^o	
26	OMRh+	BNRh+	BMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	
27	OMRh+	A ₁ BMNRh+	A ₁ BMNRh+ ^o	A ₂ BMNRh+ ^o	A ₂ MRh+ ^o	A ₂ MRh+ ^o	A ₂ MRh+ ^o	A ₂ MRh+ ^o	
28	A ₂ MNrh+	A ₁ BMNRh+	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	
29	OMNRh+	OMRh+	OMNRh+ ^q	OMRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	
30	A ₁ BMNRh+	OMNRh+	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	OMNRh+ ^o	
31	OMNRh+	A ₁ NRh+	A ₁ NRh+ ^o	OMNRh+ ^o	A ₁ BMNRh- ^q	A ₁ NRh+ ^q	ONRh+ ^o	OM	
32	A ₁ BMNRh-	A ₁ MNrh-	A ₂ BMNRh- ^q	A ₁ BMNRh- ^o	A ₁ MRh- ^q	A ₁ BMNRh- ^q	A ₁ MRh- ^q	A ₁ MRh- ^q	A ₁
33	OMNRh+	A ₂ MRh+	A ₂ MRh+ ^o	A ₁ MRh+ ^q	A ₂ MRh+ ^q	A ₂ MRh+ ^q	A ₂ MRh+ ^q	A ₂ MRh+ ^q	
34	A ₂ BMNRh+	OMNRh+	A ₂ NRh+ ^o	BMRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
35	A ₁ BMNRh+	A ₁ NRh+	BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	
36	BMRh+	OMNRh+	OMRh+ ^q	BMNRh+ ^q	OMNRh+ ^q	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	
37	A ₂ MRh+	BMRh+	A ₂ MRh+ ^q	BMRh+ ^q	BMRh+ ^q	BMRh+ ^q	BMRh+ ^q	BMRh+ ^q	
38	A ₁ MRh+	A ₁ NRh+	A ₁ BMNRh+ ^o	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	A ₁ BMNRh+ ^q	
39	BMRh+	OMNRh+	BMRh+ ^o	BMRh+ ^o	BMRh+ ^q	BMRh+ ^q	BMRh+ ^q	BMRh+ ^q	
40	A ₁ MRh+	A ₁ NRh+	A ₁ MRh+ ^o	A ₁ NRh+ ^o	A ₁ NRh+ ^q	A ₁ NRh+ ^q	A ₁ NRh+ ^q	A ₁ NRh+ ^q	
41	ONRh+	OMNRh+	OMNRh+ ^o	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	OMNRh+ ^q	
42	BMRh+	A ₁ BMNRh+	BMRh+ ^q	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	BMNRh+ ^o	
43	ONRh+	OMNRh+	ONRh+ ^q	ONRh+ ^q	ONRh+ ^q	ONRh+ ^q	ONRh+ ^q	ONRh+ ^q	
44	A ₁ MRh+	A ₂ BMNRh+	A ₁ MRh+ ^q	OMRh+ ^q	A ₁ MRh+ ^q	A ₂ BMNRh+ ^q	A ₂ BMNRh+ ^q	A ₂ BMNRh+ ^q	
45	OMNRh+	A ₁ MRh+	A ₁ MRh+ ^q	OMNRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ M
46	OMRh+	BMNRh+	OMRh+ ^q	BMNRh+ ^q	OMNRh+ ^q	OMRh+ ^o	OMRh+ ^o	OMRh+ ^o	
47	A ₁ BMNRh-	OMNRh+	A ₁ NRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
48	OMRh+	OMNRh+	OMRh- ^o	OMNRh+ ^q	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	
49	OMNRh+	OMRh+	OMRh+ ^q	OMRh+ ^o	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	
50	A ₁ BMNRh+	OMRh+	BMRh+ ^q	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	
51	BMRh+	OMRh+	OMRh+ ^o	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	BMRh+ ^o	
52	OMNRh+	A ₁ MRh+	OMNRh+ ^o	OMNRh+ ^o	ONRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ M
53	OMRh+	OMNRh-	OMRh+ ^q	OMNRh+ ^q	OMRh+ ^o	OMRh+ ^q	OMRh+ ^q	OMRh+ ^q	
54	OMNRh-	A ₁ MRh+	A ₂ MRh+ ^o	A ₁ MRh- ^q	A ₁ MRh- ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₂ M
55	OMNRh-	AMRh-	OMNRh- ^o	OMRh- ^o	OMRh- ^o	OMRh- ^o	OMRh- ^o	OMRh- ^o	
56	BNRh-	OMNRh+	BNRh+ ^o	OMNRh+ ^q	BMNRh+ ^o	ONRh+ ^q	ONRh+ ^q	ONRh+ ^q	
57	A ₂ BMNRh+	A ₁ BMNRh+	A ₂ MRh+ ^o	A ₂ MRh- ^o	A ₂ BMNRh+ ^q	A ₂ BMNRh+ ^q	A ₂ BMNRh+ ^q	A ₂ BMNRh+ ^q	
58	AMNRh-	BMRh+	AMNRh+ ^o	AMNRh+ ^o	AMNRh+ ^o	AMNRh+ ^o	AMNRh+ ^o	AMNRh+ ^o	
59	A ₁ BMNRh+	ONRh-	ONRh+ ^q	OMNRh+ ^o	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	A ₁ MRh+ ^q	
60	A ₁ MRh+	A ₁ MRh+	OMNRh+ ^q	OMRh+ ^o	OMNRh+ ^o	A ₁ MRh+ ^o	A ₁ MRh+ ^o	A ₁ MRh+ ^o	

As is apparent from the results with the families in which both parents are Rh-negative, evidently the property Rh is inherited as a dominant. In six such matings encountered in our series, all 29 children proved to be Rh-negative⁶ (This number of Rh- × Rh- families, larger than would be expected by chance, is in part due to selection of families with a known negative parent.)

In analogy to the other individual human blood properties whose heredity has been investigated, one may presume that the factor Rh is transmitted by means of a pair of genes, *Rh* and *rh*, where the dominant gene *Rh* determines its presence. Hence, three genotypes would exist, *RhRh*, *Rhrh*, and *rh rh*, the first two corresponding to the phenotype Rh+, the last to Rh-. By the usual simple calculation, one obtains from the distribution of the phenotypes in the population the frequencies of the two genes. In our random series of 448 individuals there were 69 or 15.4 per cent Rh- individuals. Accordingly, the

TABLE VI
Summary of Family Material

Mating	Number of families	Number of children		
		Rh+	Rh-	Totals
Rh+ × Rh+	42	151	7	158
Rh+ × Rh-	12	37	11	48
Rh- × Rh-	6	0	31	31
Totals	60	188	49	237

frequency of gene *rh* is $\sqrt{0.154}$ or 39.2 per cent, and the frequency of gene *Rh* 60.8 per cent. From these figures the frequencies of the three genotypes are as follows: genotype *RhRh*, $(0.608)^2$ or 37.0 per cent, genotype *Rhrh*, $2(0.608)(0.392)$ or 47.6 per cent, and genotype *rh rh*, 15.4 per cent.

From the genotype frequencies one can calculate the distribution in the offspring of various matings of the Rh factor to be expected on the hypothesis of simple dominance. In the matings Rh+ × Rh+, there are three possibilities, namely, (1) both parents of genotype *RhRh*, (2) one parent of genotype *RhRh*, the other *Rhrh*, and (3) both parents *Rhrh*. Only in case (3) can Rh- children occur. The relative frequency of this mating among Rh+ × Rh+ families is $\frac{(0.476)^2}{(0.370 + 0.476)^2}$ or 31.6 per cent. Since one-fourth of all the children of these matings should be Rh-, only 7.9 per cent of all the children of Rh+ × Rh+

⁶ Actually, in one of these families, in which blood specimens were taken from 8 children, the oldest boy was Rh positive, but on investigation it was learned that he was the child of a previous marriage. All individuals in this family were tested with human as well as with guinea pig serum.

matings should be Rh negative. The observed incidence of 7 Rh— individuals among 158 offspring or 4.4 ± 1.1 per cent, is less than the expected value, to decide whether this is accidental or significant, examination of a larger series is necessary.

In the matings Rh+ \times Rh—, two cases exist: (1) genotype *RhRh* \times *rhrlh*, or (2) *Rhrlh* \times *rhrlh*. Rh negative children will result only from the second sort of mating. The frequency of such matings among Rh+ \times Rh— families should be the same as that of genotype *Rhrlh* among Rh+ individuals, or 56.3 per cent. Hence half of 56.3, or 28.15 per cent of the children from all the Rh+ \times Rh— matings should be Rh negative. The figure observed was 11 among 48 children, or 22.9 ± 4.0 per cent, which does not differ significantly from the predicted value.

The usefulness of the blood property Rh for forensic exclusion of paternity is small, on account of the low incidence of matings Rh— \times Rh— (0.15 \times 0.15, or only 2.25 per cent), these being the only ones which permit a decision.⁷ For individual identification in medicolegal cases, determination of the Rh factor doubles the number of classifications but only fresh blood, not dried blood stains, can be tested.

Perusal of the data listed in Table V indicates that the property Rh is not a sex-linked dominant factor. This follows firstly from the equal distribution of the factor in the two sexes (cf. Table II), and secondly from the analysis of families in which the father is positive and the mother negative (families 4, 24, 53, and 59 of Table V), where on the usual hypothesis only the offspring of one sex, most probably the daughters, would exhibit the character.

Significant data relating to the question of the linkage relation of property Rh and the agglutinogens A and B are provided by family 6, and also by family 54 if one makes use of the subgroups *A*₁ and *A*. In family 6, the mother is heterozygous for both A and Rh, the father who belongs to group O, only for Rh. In this family only Rh negative children yield information as to linkage (cf. 21) that one of the two Rh negative children belongs to group A, the other to group O is evidence against close linkage. Similarly, the mother in family 54 is evidently of genotype *A*₁*A*₂*Rhrlh*, the father of genotype *OOrhrlh*. In a mating of this kind children of types *A*₂Rh+ and *A*₁Rh— belong to one category (either linked or crossover) those of types *A*₁Rh+ and *A* Rh— to the other. As there are 5 children of one sort and 1 of the other this family does not furnish evidence for close linkage.

Information as to the linkage relations of property Rh and agglutinogens M, N is

⁷ By chance such a case was encountered among a few forensic examinations in which tests for Rh were made. The test showed the following putative father BMRh—, mother, *A*₂BMRh—, child *A*₁BMNRh+. Consequently a paternity exclusion could be made from three independent results: namely, the subgroups the M, N types and the Rh factor.

provided only by families 4, 6, and 13. In family 4, three of the children belong to one class, two to the other class, in family 6 two children can be used, and one of these belongs to each class, finally, in family 13 only two of the children can be used, and again one belongs to each class. These results point strongly to independent assortment, though the possibility of a loose linkage cannot be excluded.

COMMENT

From the clinical facts mentioned in the introduction, it is apparent that a test by which Rh+ and Rh- individuals can be distinguished is of practical importance for the selection of blood donors in certain instances. This occasion arises in cases of repeated transfusion to Rh- patients, in whom the injection of Rh+ blood is sometimes harmful. The same indication obtains even at the very first transfusion in pregnancy when the woman is of type Rh-. For these reasons, where blood donor organizations exist, it will be helpful to examine the donors in order to have available a list of Rh- individuals. The fact that occasionally doubtful reactions may be encountered is of no consequence for the selection of Rh- donors, as such individuals can be excluded. To perform compatibility tests will be important, even when the donor's blood has been tested with anti-Rh sera, and should be conducted also at body temperature (22).

The test that has been described is not as perfect as one would desire, since the reactions are not strong, but with proper attention reliable results can as a rule be obtained. A favorable circumstance is the ease with which the reagent was prepared. The tests can also be made with the occasionally occurring human sera containing Rh agglutinins, but such sera are not always at hand, and unless a sufficient number of individuals previously tested for the factor are available, it may not be possible to establish the identity of a given irregular isoagglutinin with anti-Rh. Therefore, it is of value to possess in the guinea pig antisera a reagent which can be obtained at will. Moreover, indications were found of differences in the reactions of various human sera reacting on Rh+ blood, while the guinea pig sera appeared to be uniform and most likely can be used as a standard. If the agglutinable property proves to be a species character in the monkey, this could account for the uniformity of the antibodies induced by *rhesus* blood, but it has not yet been determined whether there are individual differences in *rhesus* blood with regard to the factor.

As regards the nature of the reactions described, it might be questioned whether they actually indicate a special agglutinable property or merely differing degrees of agglutinability, in view of the failure to separate Rh agglutinins from the guinea pig sera by absorption with negative bloods. The second assumption can be eliminated, however, because a separation was possible with rabbit immune sera, and the Rh agglutinins in human sera do not react at all on negative bloods and can be specifically absorbed.

The investigation of families leads one to conclude that the factor is inherited as a simple Mendelian dominant, which is not sex linked. If further studies should prove the factor to be linked to neither A,B nor M,N, as one might surmise from our own scanty data, the property Rh may serve to mark a new pair of chromosomes for linkage studies.

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SUMMARY

Studies are reported on an individual agglutinin (Rh) in human blood which has been found to be of clinical importance because occasionally it gives rise to the formation of immune isoantibodies in man, a peculiarity which leads to untoward transfusion reactions.

A method for the determination of the presence or absence of the new blood factor is described, which can be used for typing patients and prospective blood donors.

Examination of families showed that the agglutinin is inherited as a simple Mendelian dominant. The distribution of the factor Rh among white individuals and negroes may indicate racial differences. The property is probably genetically independent of the blood groups and the factors M and N.

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THE DETECTION OF A "MASKED" VIRUS (THE SHOPE PAPILLOMA VIRUS) BY MEANS OF IMMUNIZATION*

RESULTS OF IMMUNIZATION WITH MIXTURES CONTAINING VIRUS AND ANTIBODY

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The effects of the rabbit papilloma virus (Shope) are curiously complicated by the fact that the virus is oftentimes "masked" it cannot be recovered in pathogenic form from the growths produced with it in domestic rabbits (except infrequently and in small amounts) by the methods that invariably extract it in quantity from the natural papillomas of wild cottontails (1) The phenomenon may have implications for the tumor problem For the Shope virus gives rise to growths which, though benign, have the immediate characters of neoplasms, and, in addition, frequently become cancerous (2)

The general failure of attempts to "unmask" the virus has made necessary recourse to indirect methods for its detection One of these makes use of serological tests for the specific antiviral antibody This antibody is never found in the serum of normal domestic rabbits but appears in the blood of animals carrying virus induced papillomas or transplanted carcinomas derived therefrom, and its titer rises as the growths enlarge—findings which prove that the virus persists enduringly in the papillomas, as also in masked or altered form in the carcinomas, and that it increases as they proliferate (3) A second indirect method of testing for "masked" papilloma virus makes use of one of Shope's findings, namely that saline suspensions of virus induced rabbit papillomas may stimulate the production of the specific antiviral antibody when injected intraperitoneally into normal rabbits, even though they contain no pathogenic virus demonstrable by the ordinary test (4) But as a test for the presence of "masked" virus the latter method has certain limitations, which now warrant delineation

The antigenicity of different papilloma extracts varies widely, as we have repeatedly noted, those containing virus in quantity eliciting antibody in much higher titer when injected intraperitoneally into normal rabbits than others in which little or no infectious virus is present (5) Indeed, certain papilloma extracts devoid of demonstrable virus have failed, in some of our experiments, to elicit detectable amounts of antibody though repeatedly injected into normal

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rabbits,—a phenomenon not encountered by Shope (4) Does the finding indicate that virus may be absent from some papillomas, or merely that its antigenicity as well as its pathogenicity may be somehow "masked" when the growths are extracted? To learn about this we have sought to evaluate the effects of extravasated antiviral antibody upon the antigenicity of papilloma extracts For studies already reported have shown that the antibody accumulates in the papillomas in various amounts—depending upon the titer of it in the blood of the host and upon the local vascular conditions determining its extravasation—and that it is often present in quantities sufficient to neutralize or "mask" the causative virus when the growths are extracted *in vitro* (6) It seemed possible that the extravasated antibody might also reduce or abolish the antigenicity of the virus, for it had long been known that the antigenicity of other viruses and of bacterial toxins as well may be reduced or abolished upon admixture *in vitro* with antibodies directed specifically against them (7) In the work now to be reported it will become evident that extravasated antibody often influences decisively the outcome of immunization experiments of the sort outlined

Methods and Materials

The general plan of the immunization experiments was patterned closely after that of Shope (4) in order to procure results comparable with his Normal adult gray-brown (agouti) rabbits, bought from several sources outside the Institute, and weighing from 2500 to 3200 gm, were used throughout, males and females indiscriminately Groups of three or more individuals comparable as to weight were used in each experiment Crude suspensions of papillomas from wild and domestic rabbits were made by grinding the glycerolated tissue in a mortar with sand and suspending the ground paste in 0.9 per cent NaCl solution Sometimes these extracts were used as such but more often they were spun briefly to remove the sand and gross tissue debris Occasionally they were spun hard and sometimes filtered through Berkefeld candles in addition The suspensions or filtrates, sometimes after incubation with immune serum *in vitro*, were then injected intraperitoneally into the experimental animals, with precautions to prevent infection of the skin with virus¹ After an interval of 7 to 9 days the injections were repeated with freshly prepared extracts, and after a further period of about a week the animals were bled to procure serum for neutralization and complement fixation tests They were then inoculated with the virus to find whether they had become resistant to it Also they were felt all over

¹ A modification of Shope's method invariably proved effective A small slit was made through the skin with a sterile scalpel The lips of the wound were held open and the needle inserted into the subcutaneous tissue and run forward about 1 inch before it was pushed through the peritoneum After the injection had been made, slight pressure was put onto the needle with a finger as it was withdrawn, to prevent seepage along the needle track, and afterwards the wound was soaked for a minute in 5 per cent carbolic acid followed by 95 per cent alcohol

carefully and repeatedly to learn whether any papillomas had developed as might have happened had the skin become infected with the virus where the injecting needle was thrust through or elsewhere. Growths were never found and hence the results of the tests can be referred without exception to the effects of the injected materials.

The *resistance tests* were carried out by rubbing various dilutions of virus of known pathogenicity into scarified areas about 6×4 cm on the ventral skin of the injected rabbits, along with suitable normal controls, according to a procedure already described (8). Strips of fur at least 2 cm across were left between the scarified areas to prevent cross infection, and for the same reason the scarified areas were dried thoroughly by means of a blast of warm air from a small electric hair drier immediately after all of the inoculations on each rabbit had been finished. The lesions were examined as routine every 3 to 5 days from the 15th to the 42nd days and recorded as in neutralization and pathogenicity tests (8) according to a standard scale: **** = confluent papillomas, *** = semiconfluent papillomas, ** = many discrete growths, * = 5 to 15 papillomas, \pm = 2, 3, or 4 papillomas, \pm = 1 papilloma, 0 = negative. In the tables the readings are recorded on three occasions only to conserve space. They typify the findings as a whole.

The *pathogenicity tests* for virus and the *neutralization tests* for antiviral antibody were carried out as already described (8). In the former the sera to be tested were mixed with a virus filtrate of known potency and the mixtures, after incubation at 37°C for 1 hour, were rubbed into small scarified squares on the skin of three or more titration rabbits (labeled A, B, C, etc. in each experiment) which were then bandaged to prevent cross infection. The presence of neutralizing antibody in any given serum was manifest by a reduction in the number of growths appearing where the mixture of it with virus had been rubbed in as compared with those appearing as result of the normal serum or saline control inoculations.

The *complement fixation test* for antiviral antibody involves the same antigen as the neutralization test, namely, the virus, and many observations now attest to its specificity and reliability as a means of testing for the presence and quantity of the specific antiviral antibody, as also to the fact that neutralization and complement fixation are manifestations of the action of a single antibody (9). In the present work the test was carried out as previously described (9), with 2 units of complement, 2 hours at room temperature being allowed for fixation. Optimal or near optimal amounts of antigen, which consisted of Berkeley V filtrates or centrifugized extracts (water-clear) of wild rabbit papillomas containing known amounts of virus, were used to avoid post zone effects. Preliminary readings were made after 30 minutes at 37°C , and final ones after the tubes had stood overnight in the refrigerator. ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, 0 = no fixation (complete hemolysis). Control tests for anticomplementary effects were made of the materials in double amounts. These were negative in all of the experiments reported.

The comparative value of the resistance, neutralization and complement fixation tests as means of assaying the immune responses can best be perceived by the study of actual examples as e.g. Experiments 2 and 3. The resistance test is by far the most delicate of the three in the sense that it can indicate very minute immune responses. Its value is sharply limited, however, by a "low ceiling,"—meaning thereby

that resistance to the virus is complete or almost complete when a comparatively small antibody response has been elicited. Hence it is far from comprehensive. The complement fixation test, though least delicate of the three in the sense referred to above, has proved most valuable in measuring the degree of antibody response to the materials injected intraperitoneally, for in most of the experiments the serum antibody titers of the immunized animals have exceeded by far the minimum amount detectable by it, and have thus fallen into the range in which the complement fixation test is most useful. It has no "ceiling" and much experience has shown that it provides reliable quantitative results, the complement-fixing and virus-neutralizing capacities of any given serum invariably paralleling one another (9).

Effect of Serum Antibody on the Antigencity of the Papilloma Virus

As a first step towards determining the effects of extravasated antiviral antibody it seemed well to study the effect of serum antibody on the antigenicity of the virus. Various dilutions of a serum known to contain the antiviral antibody in quantity were mixed with a constant amount of a virus filtrate, and after incubation the mixtures were injected intraperitoneally into normal rabbits. They were tested as well for pathogenicity.

Experiment 1—A 1:20 virus filtrate was made as usual from the natural papillomas of cottontail 1-28, which had been preserved 8 months in 50 per cent glycerol-Locke's. A gram of the glycerolated papillomas was ground with sand, suspended in 20 cc. of 0.9 per cent NaCl solution, centrifuged at 4400 R.P.M. for 15 minutes, and the supernatant fluid filtered through a Berkefeld V candle. Filtrates previously prepared in the same way from the papillomas of this animal had invariably contained much virus. Mixtures were made of the 1:20 virus filtrate in equal parts with saline and with immune serum D.R.F. 4 in dilutions of 1:4, 1:32, and 1:128. This serum had come from a domestic rabbit with two large virus induced papillomas resulting from broadcast virus inoculation 70 days before. Previous tests had shown that it contained the antiviral antibody in large amount (titer 1:96 as determined by complement fixation). The flasks containing the mixtures were sealed with paraffin and put into the water bath at 37°C. for 4 hours and kept overnight in the refrigerator. 20 cc. of each mixture was then injected intraperitoneally as already described into each of three normal gray-brown rabbits, and the remaining portions were tested for pathogenicity in three normal rabbits. Seven days later the injected rabbits were bled from an ear vein and the sera tested for antiviral antibody by means of complement fixation.

The results of the experiment are shown in Table I. The virus-saline mixture was actively pathogenic, giving rise promptly to confluent and semiconfluent lesions in all three test rabbits, and it was highly antigenic also, eliciting antibody in all three of the rabbits injected with it, with result that their sera fixed complement completely or almost so in all dilutions up to 1:64. The mixture of virus and immune serum diluted 1:128 proved slightly less pathogenic, engendering fewer and smaller growths, and it elicited somewhat less antibody, though still much, in the three rabbits injected with it. The mixture of virus and immune serum diluted 1:32 was still less pathogenic and correspondingly less antigenic, and the mixture of virus and immune serum diluted 1:4 engendered very few growths in the pathogenicity tests and elicited com-

TABLE I
Serum Antibody Titer of Rabbits Injected with Mixtures of Virus and Immune Serum in Various Dilutions

Mixtures injected intra peritoneally	Pathogenicity of injected mixtures (Inoculation growths in test rabbits A B C)									Rabbit No.	Complement fixation titer of serum of injected rabbits†						
	17th day			26th day			36th day				1 2	1 4	1 8	1 16	1 32	1 64	
	A	B	C	A	B	C	A	B	C								
(a) Virus + Immune serum 1 4	0	0	0	0	±	0	*	±	*	13	0	0	0	0	0	0	0
										14	++	+	0	0	0	0	0
										15	++	±	0	0	0	0	0
(b) Virus + Immune serum 1 32	0	0	0	±	±	±	**	*	*	16	+++±	+++	±	0	0	0	0
										17	+++±	+++	+++	+++	0	0	0
										18	+++	+++	+++	+++	+++	±	±
(c) Virus + Immune serum 1 128	+++	±	*	++++	**	+++	++++	**	***	19	+++	+++	+++	+++±	+++	0	0
										20	+++	+++	+++	+++	+++	+	+
										21	+++	+++	+++	+++	+++	+++	+++
(d) Virus + saline	+++±	+++	*	++++	+++	+++±	++++	+++±	++++	22	+++	+++	+++	+++	+++	+++	+++
										23	+++	+++	+++	+++	+++	+++	+++
										24	+++	+++	+++	+++	+++	+++	+++

* Virus filtrate W R 1 28 1 20 mixed and incubated with equal parts of immune serum D R. F 4 in dilutions designated 20 cc of whole mixture injected into each rabbit.

† 2 units of complement

Antigen, W R 1-56, 1 120

W R = wild cottontail rabbit

D R = domestic rabbit

paratively small amounts of complement-fixing antibody in the rabbits injected with it,—so little in fact that in one instance (rabbit 13) the serum failed to fix complement at all and in the other two did so only partially, in dilutions of 1/2 and 1/4.

The results of the experiment (Table I) leave no doubt that the antigenicity of the virus filtrate was markedly reduced by admixture *in vitro* with the immune serum. The finding has been repeatedly confirmed in other experiments. Virus filtrates prepared from the papillomas of many wild rabbits have invariably proved antigenic upon intraperitoneal injection into normal rabbits, eliciting antibody in direct proportion to the amount of virus they contained, and the antigenicity of the virus filtrates, though unaffected by normal rabbit serum, has always been reduced or abolished after mixture with serum containing the antiviral antibody.

The following experiment was done to determine whether the antigenicity of the virus can be wholly abolished if the virus is mixed *in vitro* with an excess of antibody.

Experiment 2—A 1/20 virus filtrate was prepared as before of the glycerolated natural papillomas of W. R. 77, which had regularly provided much virus in previous extractions. Mixtures were made (a) with two volumes of saline, and (b) with two volumes of the serum 1/4 of W. R. 1-52, a rabbit carrying natural papillomas and having serum with a very high titer of antibody (1/512 in the complement fixation test). A third mixture (c) contained no virus but consisted of saline plus two volumes of the immune serum 1/4. All were put into the water bath for 2 hours at 37°C, and then injected intraperitoneally into normal rabbits, 6 cc. of each mixture into each of three rabbits. Eight days later fresh mixtures were made and incubated precisely as before and the injections repeated. Ten days after the second intraperitoneal injection the rabbits were bled for serum along with suitable controls, and all were inoculated with various dilutions of virus (W. R. 1-72), as already described, to test for their resistance.

At the time of the first injections supplemental tests were made to determine roughly the amount of the virus contained in the injected mixtures, and also to learn whether the mixture of virus and immune serum (b) contained antibody in excess. Mixture (a) was found to contain much virus, engendering confluent growths (****) in each of three test rabbits, the growths appearing before the 14th day. It was found furthermore that 0.2 cc. of the virus filtrate after dilution to 1/10,000 still gave rise to 2 papillomas when inoculated on the standard scarified area of two test animals. Whence it follows that 1 cc. of the 1/20 virus filtrate contained at least 5,000 infectious doses of virus. Since each rabbit in the experiment received a total of 6 cc. at each injection, it is seen that the rabbits injected with the mixture (a), which consisted of one part of the virus filtrate 1/20 and two parts of saline, received at least 10,000 infectious doses of virus at each of the two injections. The mixture (b), composed of one part of the virus filtrate 1/20, as in (a), and two parts of the immune serum 1/4, proved innocuous in the pathogenicity tests,—that is to say, the virus had been completely neutralized, and complement fixation tests of a portion of the mixture that had been

centrifugalized after incubation showed that the supernatant liquid contained practically as much free antiviral antibody as was present in the mixture (c), both mixtures fixing 2 units of complement completely in a dilution of 1/64 and incompletely in a dilution of 1/128 upon admixture with an optimal concentration of antigen.

Table II shows the results of the serum and resistance tests of the animals injected intraperitoneally. It will be noted from the complement fixation and neutralization tests that the control mixture of virus and saline (a) proved moderately antigenic, the serum of the rabbits injected with it fixing complement completely in dilutions from 1/2 to 1/16 and partially at 1/32, and the antibody was sufficient to cause complete or almost complete neutralization of a potent virus filtrate. Moreover, the rabbits injected with this mixture proved wholly resistant, or almost so to the virus, no lesions or only a few appearing where the dilutions of the test virus had been inoculated in the resistance tests. The sera of the three rabbits injected with the mixture of virus and immune serum (b) failed to fix complement at all and neutralized the virus only slightly, while in the resistance tests the injected rabbits proved almost as susceptible to the virus as the controls. The sera of the rabbits injected with the mixture of saline and immune serum (c) also failed to fix complement, but they exerted a considerable neutralizing effect on the test virus rather more indeed than the sera of the rabbits injected with mixture (b) of virus and immune serum, and the rabbits themselves proved slightly more resistant to the virus on inoculation than did the latter group.

The findings are enlightening in several ways. They disclose a fact hitherto unrealized, namely, that resistance to the papilloma virus may be conferred by passively transferred antibody. The passively transferred antibody present in the mixture of virus and immune serum (b) could conceivably account for the whole of the slight neutralizing capacity of the sera of the rabbits receiving it, as also for the resistance they manifested to the virus, this also being slight. For the mixture contained a considerable excess of antibody, as supplemental tests proved. That transferred antibody does account for the findings is indicated by the fact that the mixture of virus and immune serum (b), which presumably contained somewhat less free antibody than the mixture of saline and serum mixture (c), conferred less immunity than the latter, as the results of the neutralization and resistance tests clearly show. The conclusion seems warranted that the antigenicity of the virus, which was considerable as the results with mixture (a) proved, was completely abolished by exposure *in vitro* to an excess of immune serum.

Effects of Extravasated Antibody upon the Antigenicity of Papilloma Extracts

The findings already given have made plain the fact that serum antibody can reduce or abolish the antigenicity of the papilloma virus when mixed therewith *in vitro*. Is the same effect produced by extravasated antibody, which accumulates in various amounts in the papillomas, depending upon the titer of it in the blood and upon the local conditions influencing its extravasation (9)? There

is every reason to think so, since extravasated and serum antibody are identical (10) The results of several experiments prove the point In the first of these, two papilloma extracts were compared as to antigenicity, one containing much extravasated antibody, the other little or none Control tests were done to determine the effect of the antibody transferred passively along with one of the injected materials

Serum Antibody Titer and Resistance of

Mixtures injected intraperitoneally on 1st and 8th days§	Rabbit No	Antibody titer of serum*									
		Complement fixation tests†					Neutralization‡				
		1 2	1 4	1 8	1 16	1 32	17th day			27th day	
							D	E	F	D	27th day
(a) Virus + saline	1	++++	++++	++++	++++	±	0	0	0	0	
	2	++++	++++	++++	++++	++++	0	0	0	0	
	3	++++	++++	++++	++++	+++	0	0	±	0	
(b) Virus + immune serum	4	0	0	0	0	0	*	*	****	**	
	5	0	0	0	0	0	±	*	***	*	
	6	0	0	0	0	0	*	*	***	**	
(c) Saline + immune serum	7	0	0	0	0	0	*	0	±	**	
	8	0	0	0	0	0	0	0	**	*	
	9	0	0	0	0	0	0	0	*	±	
(d) Nil controls	10	0	0	0	0	0	±	±	**	*	
	11	0	0	0	0	0	***	**	****	****	
	12	0	0	0	0	0	**	**	****	****	
Saline control.							**	***	***	***	

* Serum procured on the 10th day after the second intraperitoneal injection

† 2 units of complement Antigen, W R 1-72, 1 20

‡ Growths resulting from mixtures of 2.5 per cent virus filtrate W R 77 E and whole serum in equal parts

§ The mixtures (a) and (b) contained at least 10,000 infectious doses of the virus, as supplemental tests showed. The mixture (b) contained a considerable excess of antibody, as was shown by supplemental tests with

Experiment 3—To procure papillomas containing much extravasated antibody, two domestic rabbits carrying vigorous, confluent papillomas were injected intraperitoneally on the 24th and 31st days after scarification with W R 1-28 virus with 10 cc. of a potent 10 per cent virus filtrate (W R 77) By the 38th day their serum antibody titers had reached 1 128 and 1 256, respectively, as determined by complement fixation tests The growths had enlarged steadily in the meantime—no unexpected finding since circulating antibody is known to be ineffective against virus in the living papillomas (8) When harvested on the 38th day the growths were char-

acteristic, vigorous, confluent papillomatous masses 3 to 4 cm across and raised 1 to 1.5 cm above the level of the skin. They were washed with soap and rinsed well to reduce the number of surface bacteria and were cut away immediately after the animals had been killed. The blood was blotted off on a sterile sponge and the connective tissue and skin trimmed away. The whole growths, including the dried keratinized parts, which presumably contained much extravasated antibody, were then diced vertically into pieces about 5 mm across and pooled and put into a single bottle of

Fixtures of Virus and Immune Serum

		Resistance to dilutions of papilloma virus											
Day	C	14th day				4th day				38th day			
		1:20	1:100	1:500	1:2500	1:20	1:100	1:500	1:2500	1:20	1:100	1:500	1:2500
0	-	0	0	0	0	±	0	0	0	±	0	0	0
0	±	0	0	0	0	0	0	0	0	0	0	0	0
0	±	0	0	0	0	0	0	0	0	0	0	0	0
*	****	****	***	**	0	****	****	***	*	****	****	****	±
*	****	****	**	±	0	**	***	**	*	****	****	****	***
±	****	****	**	0	0	***	***	±	±	****	***	±	*
±	*	**	0	0	0	***	*	0	0	****	***	±	0
0	****	**	0	0	0	***	*	0	0	****	***	0	0
*	*	**	0	0	0	***	±	0	0	***	0	0	0
*	***	***	**	*	0	****	***	***	*	****	****	****	****
**	****	***	***	0	0	****	****	**	±	****	****	****	**
***	****	***	*	*	0	***	***	±	**	****	****	****	±
***	****												

s A B C

rt.
bsorbed) supernatant fluids

sterile 50 per cent glycerol Locke's solution in the refrigerator only enough of the glycerol solution being added to cover the tissue.

The papillomas of two other rabbits provided material containing little or no extravasated antibody. The growths had been engendered with the same virus inoculum as those of the two hyperimmunized rabbits and at the same time, but were smaller and fewer, discrete and semiconfluent, 1 to 2 cm across and not over 7 mm high though fleshy and vigorous. The sera of the rabbits carrying them failed to fix complement in the standard test in dilutions of 1:2 or more and it was for this reason

that the two materials were selected. The growths were harvested as in the case of the other animals save that much of the dried material was cut away from their tops to get rid of included blood. With the same aim the diced pieces were pooled and put into about five volumes of Locke's solution for 15 minutes, with occasional stirring, and the procedure repeated with fresh fluid. The diced pieces from both animals were stored together in the refrigerator, in a considerable excess of glycerol-Locke's solution.

After 48 hours in glycerol-Locke's, the two materials were ground in mortars and suspended 1:10 in saline as usual. The suspensions were centrifugalized at very low speed for 1 minute to throw down the sand and largest tissue fragments and the turbid supernatant fluids were removed for use. The material from the hyperimmunized animals, which presumably contained much extravasated antibody, was divided into two portions for injection into normal rabbits. One portion (*a*) was injected as such, as was that from the papillomas presumably containing little or no extravasated antibody (*c*), while the other (*b*) was centrifugalized hard with the object of eliminating from it any virus or other heavy substance that might act as antigen while leaving its content of free antibody largely unaffected. Thus it would provide a control to the effects of antibody present in material (*a*). Previous work had shown that centrifugalization at 25,000 R.P.M. for 60 minutes would throw down the virus but not the antibody (9). Hence material (*b*) was spun at this speed for an hour after a preliminary spinning at 4400 R.P.M. for 15 minutes to remove coarse particles. For comparative purposes a fluid known to contain active virus was utilized. It was a 1:10 Berkefeld V filtrate (*d*) made from the glycerolated natural papillomas of W.R. 56, which were known to contain an abundance of virus. The four materials were injected intraperitoneally into comparable groups of normal rabbits, 5 cc. in each animal. Eight days later the injections were repeated, with materials prepared precisely as before. The pathogenicity of the injected materials was also tested and the capacity of (*a*), (*b*), and (*c*) to neutralize virus *in vitro*. Sera were drawn from the injected and control animals on the 16th day for antibody tests, and inoculations with various dilutions of virus were done on the 18th day to test for resistance.

The results of the tests are set down in Table III. The papilloma suspension (*a*) proved non-pathogenic, and it rendered almost innocuous the potent 1 per cent virus filtrate with which it was mixed for the neutralization test. It elicited no antibody, however, upon injection into three normal rabbits—or, at any rate, too little to be detected by the complement fixation test—though the rabbits injected with it proved partially resistant to the virus. The results were practically identical with material (*b*), which presumably had been rid of virus or other immunizing material by centrifugation but contained as much extravasated antibody as (*a*). It follows that the partial resistance to the virus manifested by the rabbits injected with the two materials can be attributed to the effects of passively transferred antibody. Material (*c*) contained no detectable free antibody, as was shown by the fact that it had no more effect on the virus than so much saline in the neutralization tests. Indeed it even proved slightly pathogenic, giving rise to 1 and 3 papillomas, respectively, on the expanses of scarified skin of two of the three rabbits of the pathogenicity test. It was notably antigenic, eliciting considerable amounts of the complement-fixing antibody in all of the six rabbits injected with it. The amount of antibody elicited was decidedly less,

however, than that called forth by the much more highly pathogenic W. R. filtrate (d) though the resistance to the virus was complete or almost so in all of the animals of both groups

The results of the experiment (Table III) may be briefly summarized. The suspension of domestic rabbit papillomas that contained much extravasated antibody (a) proved non antigenic upon injection into normal rabbits, and it conferred no more resistance than could be accounted for by the passive transfer of the antibody contained in it, as the findings with material (b) make clear. The suspension of domestic rabbit papillomas (c), which contained little or no extravasated antibody but, by contrast, a small amount of infectious virus, proved notably antigenic, though not so much so as a filtrate of wild rabbit papillomas that contained virus in abundance (d).

The findings would seem to indicate that the extravasated antibody present in quantity in one of the materials had abolished the antigenicity of any "masked" virus associated with it. It is conceivable, however, that the antigenic principle—be it "masked" virus or something else—could have been absent from this material as not from the other. A comprehensive experiment was undertaken therefore to broaden the findings. This time papilloma suspensions procured from a number of rabbits some containing much extravasated antibody and others little, were compared as to antigenicity, as in the preceding experiment.

Experiment 4—Papillomas were harvested from two groups of rabbits. One group of six rabbits carried growths engendered 40 days before with a single virus material (W. R. 56). Three of them carried confluent growths 3 to 4 cm. across and up to 1.0 cm. high and had very high serum antibody titers in consequence of two hyperimmunizing injections on the 24th and 33rd days of potent virus filtrates prepared from the pooled natural papillomas of five wild rabbits while three others had smaller discrete and semiconfluent papillomas and smaller serum antibody titers less than 1:2 in two instances. Another group of three rabbits carried confluent papillomatous masses 4 to 6 cm. across and up to 2.0 cm. high which had been engendered 142 days before with a different virus inoculum (W. R. 128). Their sera contained considerable amounts of antibody but nowhere near as much as was present in the sera of the hyperimmunized rabbits. The growths were harvested and stored in glycerol Locke's solution precisely as in Experiment 3. Those of the two rabbits with least amounts of serum antibody as determined by complement fixation were washed twice in Locke's solution further to remove any serum antibody.

The glycerolated materials were ground and suspended 1:20 in saline as usual and spun lightly to throw out sand and coarse fragments. The turbid supernatant suspensions were used for intraperitoneal injection into comparable groups of rabbits. A 1:20 Berkefeld V filtrate of the highly infectious papillomas of cottontail 1-68 was injected into another group for comparison. The rabbits to be immunized received 5 cc. of the respective materials on 2 successive days and 10 cc. of freshly prepared suspensions on the 8th day. Sera were procured for antibody tests on the 16th day and virus was inoculated immediately afterwards to test for resistance.

Table IV shows the results of the experiment. It will be seen that the papilloma suspensions procured from the hyperimmunized rabbits (63, 62, 61) contained no pathogenic virus but on the contrary had the capacity to neutralize almost completely a potent 1 per cent virus filtrate when mixed with it *in vitro*. The sera of the animals receiving these suspensions failed to fix complement in the antibody tests and the animals manifested a relatively slight resistance to the virus on inoculation as com-

Serum Antibody Titer and Resistance of Rabbits Injected with Suspensions

Materials injected intraperitoneally on 1st and 9th days*	Pathogenicity of injected suspensions			Neutralizing capacity of injected suspensions†			Rabbit No	Comp	
	24th day			24th day				1	2
	A	B	C	A	B	C			
(a) Whole suspension of D R papil lomas containing much ex- travasated antibody	0	0	0	0	0	±	52 53 54	0 0 0	
(b) Same as (a) except supernatant of suspension spun at 25 000 R.P.M. for 1 hr	0	0	0	0	0	±	55 56 57	0 0 0	
(c) Whole suspension of D R papil lomas containing little or no extravasated antibody	0	±	±	***	***	****	46 47 48 49 50 51	++++± ++++± +++++ +++++ ++ ++++±	
(d) Filtrate of W R papillomas containing much infectious virus	****	***	****				58 59 60	+++++ +++++ +++++	
(e) Nil controls							61 62 63	0 0 0	

* 10 cc. of a 1:10 saline extract injected twice into each rabbit as indicated

† Growths resulting from mixtures of the extracts indicated (centrifugalized) and 1 per cent virus

‡ Procured on the 18th day

pared with the normal controls. The papilloma suspension of rabbit 11-15, which had long carried large growths and had a considerable amount of serum antibody (titer 1:32), also failed to elicit complement-fixing antibody in the injected rabbits, and these latter proved very slightly resistant to the virus, even less so than did the rabbits injected with the papilloma suspensions of the hyperimmunized rabbits. Since the neutralization tests showed that the amount of antibody passively transferred with the papilloma suspension of rabbit 11-15 was less than that in the materials of the hyperimmunized rabbits 63, 62, and 61, and since the resistance manifested by

the rabbits injected with it was less than that conferred by the materials of the hyperimmunized rabbits, it seems likely that the slight degree of resistance manifested by all four groups of injected rabbits may have been due to passively transferred antibody. This is rendered probable also by the fact that the degree of resistance was comparable to that conferred by the passively transferred antibody of material (b) in Experiment 3 (see Table III)

Papillomas Containing (a b) Much and (c) Little Extravasated Antibody

Serum†		Resistance to dilutions of papilloma virus inoculated on 18th day											
1 32	1 64	15th day				25th day				35th day			
		1 20	1 100	1 500	1 2500	1 20	1 100	1 500	1 2500	1 20	1 100	1 500	1 2500
		0	0	0	0	±	±	0	0	±	±	0	0
		*	0	0	0	***	±	0	0	***	±	0	0
		-	±	0	0	***	***	±	±	***	***	**	±
		*	0	0	0	***	*	±	0	****		-	0
		*	±	0	0	***		±	0	****	**	±	0
		0	0	0	0	***	0	0	0	***	±	0	0
		0	0	0	0	±	0	0	0	±	0	0	0
		0	0	0	0	±	0	0	0	±	0	0	0
		0	0	0	0	±	0	0	0	±	0	0	0
		0	0	0	0	±	0	0	0	±	0	0	0
		0	0	0	0	-	0	0	0	±	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
+++	+++	0	0	0	0	±	0	0	0	±	0	0	0
+++	+++	0	0	0	0	±	0	0	0	±	±	0	0
+++	+++	0	0	0	0	0	0	0	0	±	0	0	0
		**	*	0	0	***	***	***	±	**	****	***	***
		***	***	±	0	***	***	***		***	***	***	**
		***	**	±	0	***	**	**		***	***	***	**

Saline control = same as (c) A B C = test rabbits

The papilloma suspensions coming from the rabbits with little or no serum antibody (55 and 68) had no neutralizing effect on the test virus they elicited detectable amounts of complement fixing antibody in the injected rabbits and these proved largely resistant to the virus. Similar results were got with the materials of rabbits 11 17 69 and 10-76. The suspensions of these growths contained moderate or small amounts of neutralizing antibody and they elicited various though in general small amounts of complement fixing antibody and induced but slight resistance in the rabbits injected with them.

Source of suspensions injected intraperitoneally			Pathogenicity of suspensions	Neutralizing capacity of injected suspensions§			Rabbits injected	Complement fixation			
Papillomas procured from	Antibody titer of serum	Duration of growths		28th day				1 2	1 4	1 8	
				A	B	C					
D R 63†	1 256	40	0	0	±	0	No				
							1-20	0	0	0	
							1-21	0	0	0	
	62†	1 128	"	0	0	±	0	1-22	0	0	0
								1-23	0	0	0
								1-24	0	0	0
	61†	1 128	"	0	0	±	±	1-25	0	0	0
								1-26	0	0	0
								1-27	0	0	0
11-15	1 32	142	0	±	*	*	1-28	0	0	0	
							1-29	0	0	0	
							1-30	0	0	0	
11-17	1 16	142	0	±	*	±	1-31	0	0	0	
							1-36	0	0	0	
							1-35	+	0	0	
69	1 16	40	0	**	***	**	1-37	+±	±	0	
							1-42	0	0	0	
							1-43	0	0	0	
10-76	1 32	142	0	**	**	**	1-41	±	0	0	
							1-32	0	0	0	
							1-33	+±	0	0	
55	<1 2	40	0	**	***	***	1-34	+++	++++	+++++	
							1-46	±	0	0	
							1-45	+++±	++	±	
68	<1 2	"	0	**	***	***	1-44	+++±	++++	++	
							1-39	+	±	0	
							1-40	++	++	±	
W R. 1-68			****				1-38	+++±	++++	+++++	
							1-47	++++	++++	+++++	
							1-48	++++	++++	+++++	
Normal controls.								1-49	++++	++++	+++++
								1-68	0	0	0
								1-69	0	0	0
								1-70	0	0	0

10 cc. of 1 20 suspensions of the papillomas injected on the 1st and 9th days Sera procured for test
 2 units of complement, antigen, W R. 1-28 E, 1 160

† Hyperimmunized

§ Growths resulting from mixtures of centrifugized extracts and 1 per cent virus filtrate 1 100

Resistance to dilutions of papilloma virus inoculated on 16th day												
1 128	15th day				25th day				35th day			
	1 20	1 100	1 500	1 2500	1 20	1 100	1 500	1 2500	1 20	1 100	1 500	1 2500
	***	*	0	0	****	***	±	0	****	***	±	0
	*	*	0	0	***	***	0	0	***	***	0	0
	†											
	****	***	0	0	****	****	±	±	****	****	±	±
	***	*	0	0	****	***	±	0	****	****	±	0
	***	0	0	0	****	***	±	0	****	****	*	0
	***	***	*	0	****	****	±	±	****	****	***	*
	0	0	0	0	**	*	0	0	***	±	0	0
	0	0	0	0	±	±	-	0	±	±	±	0
	****	***	±	*	***	****	***	±	****	****	***	***
	****	*	0	0	***	****	±	0	****	****	***	±
	***	***	0	0	****	****	*	0	****	****	**	±
	***	*	0	0	****	****	***	*	****	***	**	±
	†											
	†											
	0	0	0	0	±		0	0	***	*	±	0
	0	0	0	0	±	±	0	0	*	*	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	*	0	0	0	****	±	0	0	****	±	0	0
	0	0	0	0	±	±	0	0	±	±	0	0
	0	0	0	0	±	0	0	0	±	±	±	0
	0	0	0	0	*	±	0	0	±	±	0	0
	0	0	0	0	*	*	0	0	*	*	0	0
	0	0	0	0	*	±	0	0	±	*	±	0
	0	0	0	0	±	-	±	0	±	±	0	0
	0	0	0	0	±	±	0	0	*	±	0	0
	0	0	0	0	±	±	0	0	±	±	0	0
0	0	0	0	0	±	0	0	0	0	0	0	0
+	±	0	0	0	0	0	0	0	0	0	0	0
+	±	0	0	0	±	±	0	0	±	±	±	0
	***	***	*	*	***	****	***	***	****	****	***	***
	***	**	*	0	***	***	***	±	**	*	***	*
	***	***	*		***	***	***	**	**	*	***	*

The material from cottontail 1-68, which contained much virus, elicited far more antibody and far more solid resistance to the virus than any of the papilloma suspensions procured from domestic rabbits

In sum, the results of the experiment (Table IV) confirm and extend those of Experiment 3. Suspensions of the papillomas of rabbits with high serum antibody titers (D R 63, 62, 61, 11-15), which contained much extravasated antibody, proved wholly or almost wholly non-antigenic, as was shown by the fact that they elicited no antibody detectable by the complement fixation test. By contrast papilloma suspensions containing little or no extravasated antibody (D R 55, 68, 10-76, 69) proved slightly or moderately antigenic, as indicated by the amount of complement-fixing antibody present in the sera of rabbits injected with them. The various degrees of resistance to the virus manifested by the injected rabbits on test inoculation provide confirmatory findings when due allowance is made for the effects of passively transferred antibody and for the limitations of the resistance test as a measure of immunity.

The findings of Experiments 2, 3, and 4 make clear the fact that passively transferred antibody will often account for much or all of the resistance to the virus manifested by rabbits injected with papilloma extracts. And they also demonstrate the fact that the test for resistance is inferior to the serum antibody tests as a measure of the immune response to the injected materials. Enough antibody was often transferred passively to confer perceptible resistance to the virus, but under the circumstances of the experiments it was never sufficient to give complement fixation when the blood of the recipient was tested. And yet the complement fixation test is sufficiently delicate to detect comparatively small quantities of the antiviral antibody. For most practical purposes it would seem that papilloma extracts may be deemed non-antigenic if they fail to stimulate sufficient antibody to react in the complement fixation test.

Effect of Extravasated Antibody on the Antigenicity of Extracts of Cottontail Papillomas

In the following experiment the effect of extravasated antibody on the antigenicity of the virus was tested directly by mixing the two *in vitro* prior to inoculation.

Experiment 5—The glycerolated papillomas of D R 63 and 11-15 were known from Experiment 4 to contain extravasated antibody. 1:10 saline suspensions were made of them and spun at 4400 R P M for 20 minutes with removal of the clear supernatant liquids for use. Mixtures were made as indicated in Table V with a freshly prepared 1:2500 Berkefeld V filtrate of the highly infectious papillomas of W R 1-28. For comparison, two mixtures were made also with the immune serum of D R 74 (Experiment 1). The mixtures were not incubated, but were injected intraperitoneally.

into five lots of normal rabbits after they had stood at room temperature for from 5 to 15 minutes the time required to complete the manipulations. Eight days later the injections were repeated with fresh mixtures made in precisely the same way. On the 16th day the injected animals were bled and the sera tested.

From Table V it will be seen that the control mixture containing virus and saline proved moderately pathogenic, as evidenced by the papillomas produced in the test animals, and it elicited considerable amounts of antibody, as the complement fixation

TABLE V

Tests with Serums of Rabbits Injected with Virus Mixed with (a) Extracts of Domestic Rabbit Papillomas Containing Extravasated Antibody and (b) with Immune Serum

Mixture injected intra peritoneally on 1st and 8th days	Pathogenicity of injected materials						Rab bit No	Complement fixation titer of serum†				
	24th day			38th day				16th day				
	A	B	C	A	B	C		1 2	1 4	1 8	1 16	1 32
Virus W R 1 28 1 2500 5.0 cc + saline 5.0 cc	±	±	0	±±	±	±	2-04 2-05 2-06	++++ ++++ ++++	++++ ++++ ++++	0 0 ++++	0 0 ++++	0 0 ++++
Virus W R. 1 28 1 2500 5.0 cc + D R 63 pap illoma extract 5.0 cc	0	0	0	0	0	0	2-07 2-08 2-09	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Virus W R 1 28 1 2500 5.0 cc. + D R 11 15 papilloma extract 5.0 cc	0	0	0	0	0	0	2 10 2 11 2 12	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Virus W R. 1 28 1 2500 5.0 cc + immune se rum D R F 4 1 32 5.0 cc.	0	0	0	0	0	0	2 13 2 14 2 15	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Virus W R 1 28 1 2500 5.0 cc + Immune se rum D R F 4 1 128 5.0 cc	0	0	0	0	0	±	2 16 2 17 2 18	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0

† 2 units of complement
Antigen W R. 1-72, 1:120

tests proved. The mixtures containing virus plus the papilloma extracts of D R. 63 and 11:15, which contained extravasated antibody proved non pathogenic and failed to elicit detectable amounts of antibody upon injection. The mixtures containing virus plus the immune serum D R. F 4 gave similar results.

The findings of Experiment 5 show that extravasated antiviral antibody, as procured from papillomas, reduces or abolishes the antigenicity of the virus precisely as does serum antibody. Tests were next made to determine the antigenicity of extracts of cottontail papillomas containing extravasated antibody in quantity.

Experiments 6 and 7—Previous work had shown that antibody extravasates in quantity into the large confluent papillomas produced by sowing virus broadcast on the scarified skin of wild cottontail rabbits (6). To procure such growths now a 10 per cent suspension of the highly infectious W R 1-28 virus was rubbed into scarified areas about 8×10 cm on the bellies of ten normal cottontails recently trapped in Kansas. Two weeks later, three of the animals with the largest papilloma masses were selected for hyperimmunization. 10 cc of a 1:20 Berkefeld V filtrate of the infectious papillomas of W R 56 was given intraperitoneally to each, on the 14th and 21st days. On the 30th day the large, fleshy, confluent growths of two of these animals (W R 2-70 and 2-71), which were found to have serum antibody titers of 1:64 and 1:128 respectively, were harvested, diced, and placed separately in glycerol-Locke's, only enough of this being present to cover the tissue.

In *Experiment 6* a 1:20 saline suspension was made of the glycerolated papilloma tissue of W R 2-71, after it had been kept 29 days in glycerol. The suspension was spun at 2800 R.P.M. for 10 minutes and the supernatant liquid, opalescent but free from gross particles, removed for use. For comparison a 1:20 saline suspension was made in the same way of the glycerolated papillomas of W R 1-28, which were known to yield much virus. 2.0 cc of each material was injected intraperitoneally into four normal Kansas cottontails and five normal domestic rabbits, and supplemental tests were made to determine the pathogenicity of the injected materials. The rabbits were bled for serum tests on the 7th day.

Table VI shows the results of the experiment. It will be seen that the injection of the W R 2-71 material,—which proved non-pathogenic in the supplemental tests,—elicited no demonstrable antibody in either the wild or the domestic rabbits, whereas the W R 1-28 material, which contained virus in quantity, proved notably antigenic, eliciting high titers of antibody in the blood of all of the animals injected with it, roughly the same in both species.

In *Experiment 7* two intraperitoneal injections were made of the materials, which at the time of the first injection had stood 6 days in glycerol, and supplemental tests were done to determine their pathogenicity as also their capacity to neutralize added virus. A Berkefeld V filtrate of the naturally occurring discrete growths of W R 1-21 was included for comparison. Table VII shows the findings. The 1:20 crude extract of the confluent growth of W R 2-71 proved non-pathogenic, engendering no growths whatever where inoculated into three test animals. Indeed it contained an excess of extravasated antibody, as shown by the fact that it neutralized almost completely an equal volume of potent 1 per cent virus filtrate. Yet it proved antigenic, though not powerfully so. Of three rabbits injected with 2.0 cc of the material on the 1st and 8th days of the experiment, one (89) had no antibody detectable by the complement fixation test, another (90) had the barest minimum, and the third (91) had a considerable amount. Repeated injections of 5.0 and 10.0 cc of the same material elicited correspondingly greater amounts of antibody. The 1:20 crude extract of the W R 2-70 material exerted some effect on the virus in the neutralization test, though less than the W R 2-71 material.² Nevertheless it gave rise to a

² "Neutral" or near-neutral mixtures of virus and antibody often prove slightly pathogenic, as previous observations have shown. For a discussion of the phenomenon, see *The Journal of Experimental Medicine*, 1940, 72, 531.

few discrete papillomas were rubbed into the skin of the most susceptible test animal (A) though it proved non pathogenic in the other two. Injections of it elicited antibody in all of the rabbits, and the antibody titers were in general somewhat higher than those elicited by corresponding amounts of the W R 2 71 material. The W R 1 21 material, which contained much virus, was much more highly antigenic than either of the two just considered.

TABLE VI

Tests with Serum of Rabbits Injected with Extracts of Cottontail Papillomas Yielding (a) No Virus and (b) Much Virus

Source of material injected intraperitoneally	Rabbit No	Complement fixation titer of serum				
		1 2	1 4	1 8	1 16	1 32
(a) Confluent experimental papillomas of hyper immunized W R 2 71 (yielded no virus on test)	W R 1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	D R 1 50	0	0	0	0	0
	1 51	0	0	0	0	0
	1 52	0	0	0	0	0
	1 53	0	0	0	0	0
	1 54	0	0	0	0	0
(b) Discrete natural papillomas of W R 1 28 (yielded much virus on test)	W R 6	++++±	++++±	+++++	+++++	++++±
	7	+++	+++	+++++	+++++	+++++
	8	+±	+±	++++±	+++++	+++++
	10	+++++	+++++	+++++	+++++	+++++
	D R 1 55	++++±	+++++	+++++	+++++	++++±
	1 56	+++++	+++++	+++++	+++++	+++++
	1 57	+++	+++	+++++	+++	+±
	1 58	+++	++++±	+++++	+++++	+++++
	1 59	++++±	+++++	+++++	++++±	++

2 cc. of a 1:20 suspension of the papillomas was injected into each rabbit. Serum procured 7 days later for test.

2 units of complement in all tubes.

None of the sera was anticomplementary when tested concurrently in double amounts.

Antigen W R 1 72 1 120

The findings of Experiments 6 and 7 are representative of the results of several experiments made with extracts of wild rabbit papillomas that proved non pathogenic. A single injection of 2.0 cc. of the papilloma extract of W R 2 71,—which contained no infectious virus but on the contrary a considerable excess of extravasated antibody, as supplemental tests proved,—elicited no antibody detectable by the complement fixation test when injected into normal cottontail and domestic rabbits (Table VI). The same material however, elicited antibody in a subsequent experiment (Table VII) when crude suspensions of it were injected repeatedly, and so too did suspensions of another ma

TABLE VII

Tests with Serums of Rabbits Injected with Extracts of Colloidant Rabbit Papillomas Containing Little or No Demonstrable Virus

Material injected intraperitoneally on 1st and 8th days	Pathogenicity of injected materials						Neutralizing capacity of injected material†						Amount injected cc	Rabbit No	Complement fixation titer of serum									
	19th day			38th day			19th day			38th day					1 2	1 4	1 8	1 16	1 32	1 61	1 128	1 256		
	A	B	C	A	B	C	A	B	C	A	B	C												
Crude extract 1% of the confluent growth of hyperimmunized W R 2 71	0	0	0	0	0	0	0	0	0	±	0	2 0	89	0	0	0	0	0						
													90	+	0	0	0	0						
													91	++++±	++++±	++++±	±	0						
												5 0	93	+	0	0	0	0						
Crude extract 1% of the confluent growth of hyperimmunized W R 2 10													92	++++±	++++±	++++±	0	0						
													91	++++±	++++±	++++±	++++±	±						
													96	++++±	++++±	++++±	0	0						
												10 0	97	++++±	++++±	++++±	0	0						
Crude extract 1% of the confluent growth of hyperimmunized W R 2 10	0	0	0	*	0	0	±	±	±	±	*	2 0	82	++++±	++++±	±	0	0						
													80	++++±	++++±	++++±	++++±	0						
													81	++++±	++++±	++++±	++++±	0						
												5 0	83	+	++	++	++	0						
Saline control													85	++++±	++++±	++++±	++	0						
													84	++++±	++++±	++++±	++++±	0						
													86	++	++++±	++++±	±	0						
												10 0	87	++++±	++++±	++++±	+	0						
Bethesda V filtrate of the naturally occurring discrete growths of W R 1 71													88	++++±	++++±	++++±	++++±	++++±						
2 units of complement Antigen, W R 1 29, 1 120	++++	++++	++++	++++	++++	++++	++	*	±	±	±	10 0	105	++++±	++++±	++++±	++++±	++++±	++++±	0				
													104	++++±	++++±	++++±	++++±	++++±	++++±	++++±	++++±	0		
													106	++++±	++++±	++++±	++++±	++++±	++++±	++++±	++++±	++++±	±	

2 units of complement

Antigen, W R 1 29, 1 120

† Growth resulting from mixtures of papilloma suspensions and 1 per cent virus filtrate W R 1-21 in equal parts

terial of similar sort. But both materials proved far less antigenic than cottontail extracts containing virus in quantity.

It is noteworthy that extracts of wild rabbit papillomas, even when they contained little or no infectious virus as in Experiments 6 and 7, have invariably proved more highly antigenic than those of domestic rabbit papillomas.

COMMENT

The experiments here reported were undertaken to learn why extracts or suspensions of some domestic rabbit papillomas fail to elicit antiviral antibody upon intraperitoneal injection into normal rabbits. The finding had been exceptional in our experience and Shope had not encountered it (4). Yet it seemed to bear upon the phenomenon of the "masking" of the virus in domestic rabbit growths, and to mean either that the virus is absent from some papillomas as not from others, or that something is present in certain growths that renders extracts of them non antigenic. Extravasated antiviral antibody is often present in the papillomas and cancers of wild and domestic rabbits in amounts sufficient to neutralize and effectively "mask" any virus that might be liberated when the growths are extracted *in vitro* (6), and it seemed likely that extravasated antibody might reduce or abolish the antigenicity of the virus. This proved to be the case. Saline suspensions of domestic rabbit papillomas that contained much extravasated antibody proved completely or almost completely non antigenic upon injection into normal rabbits, while suspensions or extracts of comparable growths that contained little or no extravasated antibody were invariably antigenic in concurrent tests.

The findings disclose certain limitations in the value of immunization experiments as a means of demonstrating "masked" virus. Extravasated antibody may complicate the outcome of such experiments in either or both of two ways. Firstly, antibody transferred passively with the injected material often confers resistance to the virus (Experiments 3 and 4), and this may be confused with an immune response on the part of the injected animal. For this reason the positive results of such experiments require careful interpretation. Secondly, negative results are often due to the fact that extravasated antibody has reduced or abolished the antigenicity of the virus present in the injected material (Experiments 3, 4, and 5). Since the antiviral antibody is almost invariably present in greater or lesser amounts in extracts of virus induced papillomas, as also in extracts of the cancers derived from them (6), the value of immunization experiments as a means of demonstrating "masked" virus in such growths would appear to be restricted to the instances in which positive results are got, and then only when the effects of passively transferred antibody can be ruled out. This can be done when considerable amounts of serum antibody are called forth. Obviously, a negative result can have no certain meaning if extravasated antibody was present in undetermined quantity in the injected material. For it may mean either that the antigenicity of the virus had been abolished

by extravasated antibody, or that the virus was present in amounts too small, or in a form which did not stimulate detectable amounts of antiviral antibody, or that it was actually absent

Mention should be made here of the negative results of immunization experiments with extracts of cancers deriving from the papillomas of cottontail rabbits. Syverton reported that he had failed to elicit detectable amounts of antibody upon injection of cottontail cancer extracts into normal rabbits (11), and Dr. Peyton Rous and I got the same result in similar, unpublished experiments. Our further tests showed that antibody extravasates in great quantity into cottontail cancers (12). Whether this was the sole reason for failure of the immunization experiments cannot now be said, since the amount of antibody which had come out into the growths was not determined, though it was noted to be present in such quantity in some cases that the cancer extracts proved capable of neutralizing added virus. Certainly the latter persists in masked or altered form in the cancers deriving from the papillomas of domestic rabbits (3), and much evidence indicates that a variant of it is responsible for the cancers of cottontails (12).

A fact discerned in previous work (5) and strikingly illustrated in the experiments of the present paper deserves special emphasis here, namely, that extracts of domestic rabbit papillomas are in general much less antigenic than extracts of wild rabbit growths. For example, crude suspensions of the most potent domestic rabbit materials (Experiments 3 and 4) elicited scarcely as much antibody as did a Berkefeld filtrate of a cottontail growth diluted 1:2500 (Experiment 5). The finding fits in with the observation that the antibody titer attained in the blood of domestic rabbits carrying virus-induced papillomas is in general much lower than that in cottontails with growths of comparable size and duration (5). Does it provide a clue to the phenomenon of the "masking" of the virus in domestic rabbit papillomas? Can the comparatively slight antigenicity of suspensions of domestic rabbit papillomas mean that they contain a relatively small amount of virus,—an amount too small in most instances to be detected by the inoculation methods now in use? Or does the finding indicate that the virus is somehow altered, both antigenically and pathogenically, in domestic rabbits, a species to which it is foreign? As concerns the state of the "masked" virus in the papillomas of domestic rabbits, the fact may be mentioned that the antigenic principle present in them appears to elicit antibody of precisely the same sort as that called forth by the naturally occurring virus (9), though in much lower titer, as already mentioned. Furthermore, it would seem to be acted upon by the antibody in precisely the same way: the results of Experiments 3 and 4 make plain the fact that the antigenic principle in domestic rabbit papillomas is rendered non-antigenic by extravasated antibody precisely as is the virus. At first sight this finding would seem to conflict with two facts previously noted, namely, that extracts of domestic

rabbit papillomas fail as a rule to fix complement perceptibly upon admixture with the antiviral antibody, and that they fail also to absorb detectable amounts of the latter *in vitro* (9). But considerable quantities of the virus are required to consummate the *in vitro* serum reactions,—we have never observed complement fixation or antibody absorption with virus filtrates diluted more than 1:1280,—and the amount present in domestic rabbit papilloma extracts may be too small. All of the facts are compatible with the assumption that much less virus is present in the papillomas of domestic rabbits than in those of cottontails, its natural host.

SUMMARY

A study has been made of the immunization procedure described by Shope, with particular reference to the detection of "masked" papilloma virus by means of it. Papilloma extracts were frequently encountered which, though non pathogenic, elicited the specific antiviral antibody and induced resistance to the virus upon injection intraperitoneally into normal rabbits. The results of the immunization experiments were often complicated, however, by the effects of extravasated antibody which had accumulated in various amounts in many of the papillomas and was consequently present in extracts of them together with "masked" virus. The extravasated antibody was often sufficient to render extracts of domestic rabbit papillomas non antigenic, and sometimes, when present in excess, its passive transfer conferred resistance to reinfection with the virus. The conclusion seems warranted that only positive immunization findings can be interpreted with certainty. Negative results provide no decisive evidence as to whether "masked" virus is or is not present in the injected material, unless the amount of extravasated antibody also present is known. The findings may have a bearing on the negative outcome of immunization experiments with extracts of the cancers deriving from the natural papillomas of cottontails.

Crude suspensions of domestic rabbit papillomas, which contain little or no virus demonstrable by ordinary methods, are far less antigenic than extracts of the natural growths of wild rabbits, which contain virus in quantity. In explanation of the finding the possibility seems worthy of attention that domestic rabbit papillomas may contain much less virus than the growths of cottontails, the natural hosts of the virus.

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EXPERIMENTAL NEPHRITIS IN RATS INDUCED BY INJECTION OF ANTIKIDNEY SERUM

V CHRONIC NEPHRITIS OF INSIDIOUS DEVELOPMENT FOLLOWING APPARENT RECOVERY FROM ACUTE NEPHROTOXIC NEPHRITIS*

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PLATES 14 AND 15

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Previous experiments have demonstrated that the acute glomerulonephritis induced in rats by the injection of anti rat kidney serum prepared in rabbits will, under proper conditions, develop into a chronic progressive disease with ultimate renal failure and death (1). Early in the work we believed that the principal factor influencing the development of chronic progressive nephritis was the quantity of nephrotoxin administered. Thus, the injection of an appreciable quantity of a weakly nephrotoxic serum, or of a small amount of a more potent antikidney serum, resulted in a mild nephritis that rapidly subsided. Continued renal irritation was originally observed only in rats that had survived a severe acute disease induced by sublethal amounts of nephrotoxin. Later, however, it was shown that the course of the nephritis could be markedly influenced by diet (2). For example, almost all of the rats fed a basal diet after receiving adequate amounts of nephrotoxin developed chronic nephritis and the majority died within a year with renal insufficiency. On the other hand the feeding of a high protein diet to similarly injected animals uniformly resulted in progressive kidney involvement, and all but a few died within a matter of months. Finally rats fed a low protein diet almost invariably recovered promptly from the acute process. More recently (3), an additional factor, *etc*, one inherent to differing degrees in certain inbred lines of rats, was found to affect the course of the induced disease. The purpose of the present study was to amplify the observations on the rôle of heredity in the experimental glomerulonephritis that follows injection of antikidney serum.

Materials and Methods

Strains of Rats—*Wistar-Kyoto Strain*. This inbred strain of animals was used exclusively throughout the earlier studies. Its origin remains obscure. The animals practi-

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cally all of which are hooded, were raised as pets for a number of generations. They were healthy, easy to handle, free of scabies and had a low incidence of infection with *Salmonella muris*, in the beginning, these were the principal reasons for employing this strain of rats.

Long-Evans Strain Rats of this breed were descended from animals left at the Institute by Dr Herbert M. Evans.¹ These animals, like the Whelan rats, were generally hooded, but the adults were definitely heavier and of greater stature than Whelan animals.

Wistar Strain Animals employed in this work were raised from a group of rats of the "Experimental Colony Strain" obtained from the Wistar Institute in 1937.

Anti-Rat-Kidney Serum—Nephrotoxic serum was obtained from rabbits immunized with suspensions of perfused rat kidney (4). Antiserum pool I was prepared by injecting renal tissue from animals of the Whelan strain and was administered intravenously to rats of the three strains when they attained a fasting body weight of 60 to 80 gm.

Feeding and Care of Rats—Two isocaloric diets were employed. These have been previously described in detail and designated basal and high protein diets (2). They contained, respectively, 18 and 40 per cent of protein and 51 and 29 per cent of carbohydrate, fats, minerals and vitamins were present in each diet mixture in similar amounts. Animals were kept in individual glass cages with food and water constantly available. The methods used for collecting and examining urine have been described in earlier publications. Rats were weighed and their urine specimens were analysed on alternate days during the first few weeks after injection of anti-kidney serum, subsequently these data were collected once or twice a week.

Rats were sacrificed when moribund or at the end of designated periods of observation. Organs were fixed in acetic Zenker's solution and paraffin sections prepared and stained by the usual methods (1b).

EXPERIMENTAL

Throughout most of our work on nephrotoxic nephritis, rats of the Whelan strain have been employed. However, in recently reported experiments (3), animals of the Whelan and Evans strains were injected with comparable amounts of anti-rat-kidney serum. Evans rats, in contrast to the Whelan animals, showed a marked tendency to recover from the acute renal injury even when maintained on a high protein diet. This unforeseen development, in an experiment designed for the elucidation of another point and terminated at the end of 3 months, seemed to warrant further investigation.

¹ Dr Evans, in a personal communication, informs us that this strain of rats originated in 1910 in the laboratory of Professor Joseph A. Long of the Department of Zoology, University of California, Berkeley. Two albinous females of unknown origin were mated with a wild gray male rat which was trapped in the vicinity and probably was of the Norwegian strain. Brother-sister inbreeding of the two litters which resulted gave the Long-Evans strain. Throughout this paper we shall designate these animals as Evans rats.

Course of Nephritis in Rats of the Whelan, Evans and Wistar Strains—The response of rats of three different inbred lines to injections of nephrotoxin and the subsequent course of the resultant nephritis were studied in the following experiment

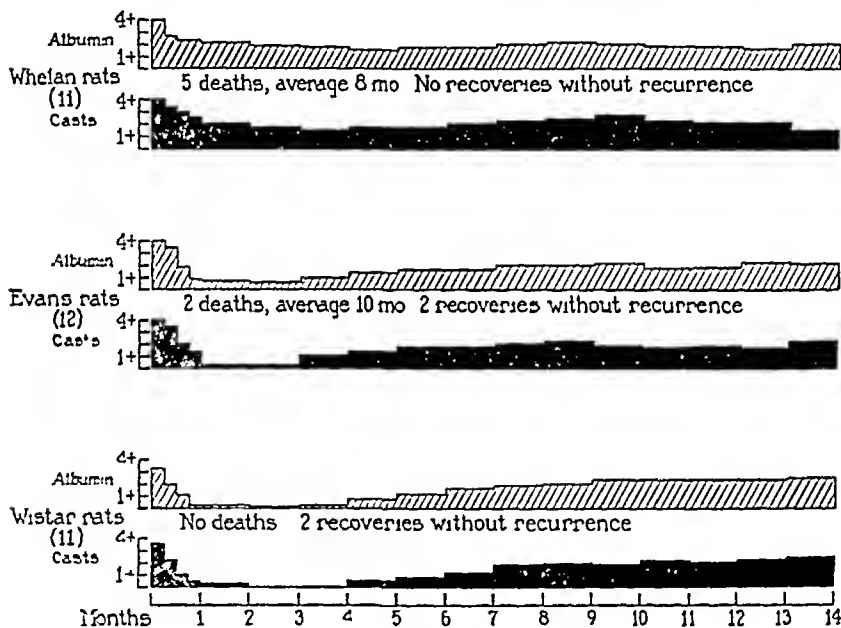
Groups of twelve young rats of comparable weight and sex, of the Whelan Evans and Wistar strains respectively were injected with a total of 10 cc. of antikidney serum, pool I, per 100 gm body weight in divided doses over a period of 3 days. This antiserum which had been prepared by immunizing rabbits with renal tissue from Whelan rats was capable of inducing moderately severe acute nephritis in Whelan animals injected in this manner. One half of the members of each group was fed a basal diet the remainder received a high protein diet. Observations were continued for 14 months when the surviving rats were sacrificed. One of the Whelan animals was discarded because of improper injection of nephrotoxin. A Wistar rat, which died following an accident on the 90th day of observation, is not included in the group. A difference in the severity of the chronic nephritis was noted in animals of the Whelan strain fed the basal and high protein diet. However, the response of rats of the other two strains was similar on both diets. For simplicity, the data presented in Text fig. 1 have been arranged to portray graphically the average excretion of protein and of casts by animals of each of the three strains without regard to diet.

The acute nephritis induced in Whelan rats of the present group was somewhat less severe than that observed earlier in animals of this strain that received a smaller amount of a more potent antiserum (2). None of the eleven Whelan animals died from renal failure associated with the acute nephritis but all developed anasarca during the 1st week after injection. The milder degree of initial renal injury undoubtedly contributed to the prolonged course of the chronic nephritis, e.g., six of the eleven Whelan rats lived through the 14 months of the experiment and these survivors were evenly divided between the two diet groups. Albuminuria and cylindruria, which were 4+ during the week following injection of nephrotoxin, diminished thereafter but remained between 3+ and 2+ throughout the period of observation (Text fig. 1). None of the animals recovered permanently. One rat in the basal group excreted normal urine between the 2nd and 4th months but during the remaining months had urinary changes indicative of low grade to moderate renal involvement. Five rats died with chronic nephritis, their average life span after injection was 8 months.

Rats of the Evans strain responded to nephrotoxin with an acute renal injury almost as severe as that of Whelan rats, excretion of albumin and casts was comparable in the two groups but anasarca was not observed among the Evans animals. Moreover, urinary abnormalities rapidly diminished after the 2nd week in members of this group. Indeed, from the 30th to the 70th days the urines of the majority of the rats were free of albumin and casts, and in the remainder only relatively slight amounts of these constituents were

present Following this period of apparent recovery or of low grade nephritic activity, one after another of these rats began to excrete progressively increasing amounts of albumin and casts By the end of the 6th month, the average urinary abnormalities of the Evans group were comparable to those of the Whelan group, this was in spite of the fact that two animals recovered shortly after injection and remained free of signs of nephritis thereafter Only two of the Evans rats died with chronic nephritis, these animals, a male and a female were both fed a basal diet (see Text-fig 1)

Urinary abnormalities shown by three strains of rats
after induction of nephrotoxic nephritis



TEXT-FIG 1

Wistar animals behaved even more atypically than Evans rats in their response to nephrotoxin when compared with the behavior of members of the Whelan strain as a standard In the first place, the excretion of protein and formed elements during the 1st week after injection, while marked, was less intense than in the other two groups Furthermore, subcutaneous edema was not observed during the acute phase of the induced nephritis Finally, the urinary abnormalities diminished so rapidly during the 1st month after injections that at the end of this period, half the animals had normal urines and the remainder excreted only traces of albumin and a few casts During the 3rd month, eight of the animals were considered to have recovered completely

while two were regarded as being in a latent phase, the final animal regularly excreted small amounts of albumin but no casts during this period. The two rats with latent nephritis gave evidence of increasing renal irritation during the 4th month. Furthermore, like the Evans rats, the Wistar rats which had been considered cured also developed recurrences, until by the end of the 7th month all but two of the animals were suffering from active chronic nephritis. The two rats just mentioned remained normal throughout the remainder of the period of observation. Recurrence of kidney disease, once established, progressed slowly but unremittingly (Text fig 1). The general appearance of the rats remained good and none died during the 14 months of study.

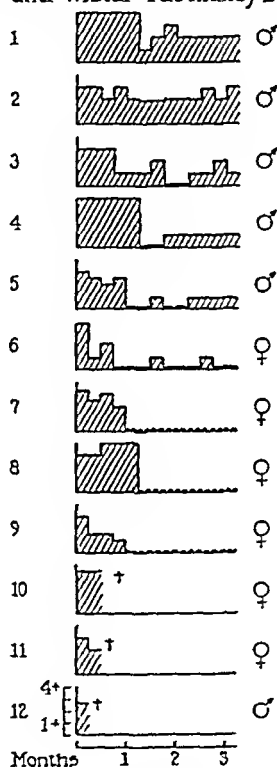
Rats of the Evans and Wistar strains irrespective of the diet consumed, followed the same general course: a rather rapid amelioration of the acute nephritis followed by a period of several weeks or months during which the nephritis appeared to be cured or in a state of latency, and finally, a recrudescence of renal disease which was slowly progressive. It may be mentioned, although the interpretation is not immediately obvious, that the two Evans and two Wistar rats (a male and a female of each strain), which recovered without recurrence of nephritis were all fed a high protein diet. Furthermore, recurrences appeared somewhat later and were slightly less severe in the animals fed a high protein diet. This is in contrast to the observation on Whelan rats in which signs of renal injury were greater in the animals given a high protein diet.

Nephritis in Rats of the Wistar and Whelan Strains Induced by Anti Wistar Rat Kidney Serum—Observations on the severity of acute nephrotoxic nephritis and the apparent recovery from the initial disease which are recorded in the previous section are subject to at least two interpretations. In the first place, animals of the several inbred lines may differ in their susceptibility to nephrotoxin and in their tendency to recover from its injurious effects because of inherited characteristics. On the other hand, the variable responses might depend primarily upon differences in the effective potency of the nephrotoxin rather than upon the susceptibility of the animals. For example, if the renal tissues of each inbred line were of slightly different antigenic structure, then the nephrotoxic antibody obtained by immunization with tissue from one line might be most active against the kidneys of animals of the homologous strain. This idea was investigated by preparing anti Wistar rat kidney serum and observing its effect on animals of the Wistar and Whelan strains.

Preliminary titrations indicated that antiserum 4733, which was prepared by immunizing rabbits with suspensions of perfused kidney tissue from Wistar rats, induced only a mild and transient renal disease in young rats of the Wistar strain that received 10 cc per 100 gm of body weight whereas the administration of 14 cc. was followed by severe kidney damage. Groups of twelve Wistar and Whelan rats of comparable age, weight and sex were injected with antiserum 4733 in the following manner: twelve Wistar rats received intravenously on consecutive days 0.2, 0.4 and

0.8 cc of antiserum per 100 gm body weight, six Whelan rats were similarly treated, and finally, six other Whelan rats were injected with 0.2, 0.4 and 0.4¹ cc of antiserum per 100 gm body weight during the same period. All the animals were fed a high protein diet. Survivors were observed in the usual manner for 3 months. The degree of albuminuria displayed by individual animals in the Wistar group is graphi-

Albumin excreted by 12 Wistar rats
after injection of 1.4 cc. per 100 gm body weight
of anti-Wistar-rat-kidney serum



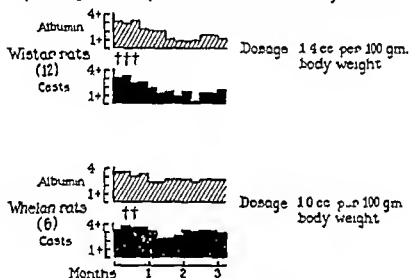
TEXT-FIG 2

cally portrayed in Text-fig 2. The average excretions of protein and formed elements by members of the first and last groups are presented in Text fig 3.

All animals of the Wistar strain receiving 1.4 cc amounts of serum 4733 developed severe acute nephritis. On the day following the third injection of antikidney serum marked oliguria was observed, the urines coagulated solidly when heated with dilute acetic acid or when mixed with 10 per cent trichloroacetic acid; however, they contained only a few epithelial cells and cellular casts and failed to give a positive guaiac test. During the ensuing week

the degree of proteinuria diminished slightly but all the rats developed marked cylindruria and anasarca. Three animals died of renal failure during the first 2 weeks. The clinical course of the induced disease in surviving animals was irregular, as is apparent from the data presented in Text fig 2. Two rats displayed signs of severe renal involvement throughout the period of observation. Three animals improved considerably after the 1st month but continued to show evidence of moderate kidney disease until sacrificed. Three rats had apparently recovered completely by the end of the 1st month. The final animal in the group improved rapidly but intermittently excreted small amounts of albumin and casts. In this group of Wistar animals that survived the acute injury, sex apparently influenced the course of nephritis for all of the males

Urinary abnormalities shown by two strains of rats after injection of anti-Wistar rat kidney serum



TEXT FIG 3

developed severe or moderate chronic nephritis while three of the four females apparently recovered and the other presented only slight urinary abnormalities during the late part of the experiment. It is doubtful whether any significance can be attached to the observation that two females, but only one male, succumbed with acute nephritis.

The same dose of nephrotoxin 4733 that had been administered to the Wistar rats, i.e., 1.4 cc per 100 gm body weight, was uniformly lethal for members of the Whelan strain: all animals developed severe nephritis and succumbed between the 6th and 12th days following the third injection of antikidney serum. Even the injection of 1.0 cc of this serum in Whelan animals resulted in nephritis of such severity that two of the six rats died during the 2nd week. Each of the surviving animals continued to excrete highly abnormal urine throughout the 3½ months of observation. The results of urinalysis summarized in Text fig 3, indicate the severity of the chronic nephritis in this group of animals and bring into contrast the degree of average involvement in Whelan rats receiving

10 cc of nephrotoxin with that of Wistar animals injected with a larger amount of antiserum

It is apparent from the evidence so far presented that the difference in intensity of acute nephrotoxic nephritis induced in animals of the Wistar and Whelan strains by a given dose of antikidney serum is dependent primarily on the relatively different vulnerability of the kidneys of members of each inbred line and not on strain specificity of the nephrotoxin. The experiment brings out an additional fact, namely, the difference in the ability of rats of the two lines to recover, temporarily at least, from the injury induced by nephrotoxin. Thus, Wistar rats with severe acute nephritis tended to recover even while being fed a high protein diet. Whelan animals with a correspondingly intense acute nephritis have never recovered while being maintained on a high protein diet. Finally, for the first time in our experience with nephrotoxic nephritis, sex appears to have had a consistent influence on the course of the disease. It is evident from the data presented in Text-fig 2, that female Wistar rats recovered more promptly and completely from the acute injury than did male Wistar rats.

Pathological Changes in the Kidneys of Different Strains of Rats—Histopathological lesions observed in sections of kidney tissue from Whelan rats used in the current experiments were similar to those described earlier (1, 2). Moreover, essentially identical renal lesions were encountered in Wistar and Whelan rats that died of acute nephritis 1 to 2 weeks after injection of anti-Wistar-rat-kidney serum. In animals of both strains, the glomerular tufts were comparatively large but relatively anemic; marked uniform thickening of the glomerular capillary basement membranes was mainly responsible for the enlargement; in addition, some increase in number of endothelial nuclei and swelling of epithelial cells contributed to the size of the tufts. Tubules observed in the kidneys of Whelan and Wistar rats with acute nephritis were moderately dilated, their lumina contained albuminous material and their epithelial cells showed various stages of degeneration. Fig 1 illustrates the typical severe acute renal lesion in a Wistar rat.

The renal lesions encountered in Wistar rats, Nos 1 and 2, that showed clinical signs of moderately severe nephritis throughout the 3 months of observation, were practically indistinguishable from those of the four Whelan rats that received the same anti-Wistar-kidney serum and survived the 3 months period of the experiment (Figs 4 and 5). Sections from these animals showed extensive glomerular and tubular lesions. Varying degrees of distortion and scarring of the glomerular tufts were brought about by irregularly thickened glomerular capillary basement membranes, which were not infrequently frayed and sometimes had given rise to intracapillary fibrillae; distortion had also been induced by ingrowth of connective tissue cells from the stalk or from crescents that had partially obliterated the capsular space.

Groups of tuhules with thickened hasement membrane, some with collapsed lumina and others with dilated empty lumina, were enmeshed in connective tissue which often contained foci of lymphocytes. Still other tuhules were moderately dilated and filled with precipitated protein, or occasionally with hyalin casts. Hypertrophy of epithelium such as occurs in normal rats fed a high protein diet was present in many tuhules hut, here and there in a proximal segment, hyperplasia of epithelial cells had resulted in partial or complete occlusion, frequently epithelial cells in such areas had undergone necrohiotic changes and occasionally were even necrotic.

Of special interest were the histological findings in the kidneys of Wistar rats that had apparently recovered from the effects of anti Wistar kidney serum and that were sacrificed 3½ months after injection. Examination under low magnification revealed few ahnormalities except a rare renal unit which was fibrotic (Fig 3). Residual glomerular changes were discernihle, however, in all of the Malpighian bodies in sections stained hy the Mallory Heidenhain technique and examined under higher magnification. These were uniform moderate dilatation of glomerular capillaries and widely distributed irregular thickenings of moderate degree of the capillary hasement membranes (Fig 2). Usually each tuft contained one or more strands of new formed connective tissue which had grown into the lohules from the stalk. Occasionally slight thickening of the capsular hasement membrane was observed, and rarely adhesions joined the tuft and capsular epithelium, but crescent formations were not seen. None of these glomerular changes was conspicuous in sections stained with hematoxylin and eosin. Some hypertrophy of tuhular elements was encountered such as is characteristically present when the high protein diet is fed. It may also be pointed out that lesions similar to those just described were also found in the renal glomerular tufts of Evans rats which had made a complete or nearly complete clinical recovery while consuming either high or low protein diets and which were sacrificed 3 months after receiving markedly toxic doses of antikidney serum (3). In brief, clinical recovery from nephrotoxic nephritis, whether occurring in Whelan or Evans rats fed a low protein diet (2, 3) or in Evans (3) or Wistar rats maintained on a high protein diet, was characterized histologically hy almost complete return to normal of tuhular structures hut hy the persistence of definite though minor glomerular fibrosis.

Histopathological studies on Whelan, Evans and Wistar animals that survived 14 months after receiving anti Whelan rat kidney serum contributed relatively little to an understanding of the pathogenesis of the recurring nephritis which developed after a period of apparent latency or recovery. The renal lesions in rats of all three strains with chronic nephritis were so alike that it was often impossible to tell from which group a given section had originated. In general, however, the pathological process was more severe in members of the Whelan line (compare Figs 7 to 9), this was not unexpected, since the dis

ease terminated fatally in almost half the animals in this group. It is also apparent from Figs 11 to 13, that while qualitatively the glomerular and tubular scarring were similar in animals of the three groups, the number of completely fibrosed glomeruli and the amount of crescent formation were slightly greater in the Whelan rats. It seems reasonable to assume that the chronic nephritis observed 11 to 14 months after injection of antikidney serum represents the late result of a progressing disease of the type observed in rats 3 months after injection and illustrated in Figs 4 and 5. Nevertheless, it is evident from the protocols that the animals whose kidneys are illustrated in Figs 11 and 12 excreted essentially normal urines 3 months after injection, hence they may be considered to have had at that time mild lesions corresponding to those illustrated in Figs 2 and 3. Is it not possible that the comparatively mild damage present in kidneys that have apparently recovered on clinical grounds may at a future time serve as a basis for the development of a slowly progressing lesion? Indeed, the glomerular changes did increase in intensity even in those Evans and Wistar rats that remained clinically free of disease for as long as a year after recovery. It is obvious that the amount of intraglomerular scarring is greater in Fig 10 (14 months) than in Fig 2 (3 months). In fact, the tuft lesions observed in the former are almost as severe as those found in many of the Malpighian bodies of rats with clinically demonstrable chronic progressive nephritis sacrificed 3 months after injection (Figs 4 and 5), nevertheless, significant tubular changes are not seen (Figs 6 and 10).

DISCUSSION

The various types of renal disease that are caused directly by antikidney serum or that take their origin from the injury induced by nephrotoxin are about as manifold as are those types grouped under the term "Bright's disease," in man. For, in addition to an acute glomerulonephritis which can be directed toward either permanent clinical recovery or progressive chronic nephritis terminating in uremia (2), we have also induced acute renal injury which quickly subsides, as shown by urinary examination, leaving the rats apparently physically normal and with only slight residual histopathological changes in their kidneys. Nevertheless, after a few months time, animals of this latter sort develop by insidious onset a chronic nephritis which is slowly progressing. These several forms of the experimental disease have been obtained by varying two factors, namely, heredity (Whelan, Evans and Wistar strains of rats) and diet (low, basal and high protein diets). There is some indication in the present work that sex also may influence the course of the disease.

It seems justifiable in light of the present experiments to differentiate between two phases in the response of animals of the several inbred lines to neph-

rotoxin These are, first, relative susceptibility to nephrotoxic injury and, second, comparative capacity to recover from such an induced injury The order of increasing susceptibility of the three strains to nephrotoxin was Wistar, Evans and Whelan, with only a slight difference between the last two, but an appreciable disparity between them and the Wistar strain Similarly, the decreasing order of the three as regards their capacity to recover from the initial nephrotoxic trauma was Wistar, Evans and Whelan Thus, under similar environmental conditions, Whelan rats were most susceptible to nephrotoxic injury and least capable of repairing the damage once it was established, the converse was true for Wistar rats It would appear that both aspects of response to nephrotoxin, i.e., vulnerability and capacity to recover are conditioned by some trait inherited by rats of different inbred lines Furthermore, the latter aspect can be influenced by an environmental condition such as diet, or rather by the comparative amount of functional injury that probably results from varying diet Whether relative vulnerability to nephrotoxic injury can also be altered by diet has not been studied The importance of inherited factors in affecting resistance is well established from work with cancer and with infectious agents Furthermore, Chase has recently shown that susceptibility to sensitizing chemical agents is also affected by heredity (5) The influence of heredity on the capacity of damaged tissue to undergo restoration is not so clearly understood It is interesting to recall the experiments of MacNider (6) which show a divergent susceptibility to injury of tissues of animals of different age groups and which, furthermore, demonstrate the tendency of young animals to replace injured cells with normal ones, whereas older animals either fail to initiate repair or restore the structure with atypical cells

The period of recovery from acute nephritis followed by the gradual development of chronic nephritis in Evans and Wistar rats fed a basal or high protein diet suggests strongly that different mechanisms may be responsible for the acute and chronic renal disease that develop after injection of antikidney serum It was and still is difficult to understand how antibody from a heterologous species, i.e., nephrotoxic rabbit serum, could persist in the rat's body for 3 to 12 months and contribute to the progression or recurrence of renal disease Because of this, we spoke in an earlier paper (1b) of "chronic nephritis that originates in the acute damage induced—by nephrotoxin" and merely said that "renal lesions of the early phase merge into scarring of the glomeruli and tubules" It seems probable that nephrotoxin causes only the acute renal damage and that subsequent pathological changes (except simple connective tissue replacement of structures totally destroyed by acute injury and some residual thickening of glomerular membranes) arise as a result of a slightly diseased organ attempting to function under conditions which are unfavorable If such is the case then it might be expected that in Whelan rats which are most susceptible to nephrotoxin and least able to recover from its effects, the

two processes responsible for the acute and chronic nephritis respectively might so overlap that the limits of each become indistinguishable. In Evans and Wistar animals, on the other hand, the two processes seemed to be separated by an interval of apparent clinical recovery, and hence each process is clearly distinguishable from the other.

A question of considerable interest is concerned with the fundamental damage caused by acute nephrotoxic nephritis, which usually leads sooner or later to the development of chronic nephritis. There is ample histopathological evidence of slight scar formation in the glomerular capillaries of most rats that have suffered from the acute disease. Moreover, this small amount of scarring in each glomerulus multiplied by the total number of glomeruli undoubtedly results in a diminution in total functioning glomerular tissue, notwithstanding the fact that function, as estimated by urea clearance, may be normal during the period of recovery (2). In this connection one might recall the opinions of Medlar and Blatherwick (7), whose description of dietary nephritis in partially nephrectomized Wistar rats conforms closely on clinical and pathological grounds with our observations of recurrent nephritis in Evans and Wistar rats. These authors have suggested that the development of progressive nephritis "hinges on the production of irreparable damage to the filter bed of the glomerulus—and that the etiological factors initiating this primal damage may be multiple and diverse in character." In agreement with this point of view is the fact that practically all histopathological alterations which are detectable in the kidneys of Evans and Wistar rats during the period of clinical recovery from acute nephrotoxic nephritis are limited to the glomerular filter bed. It is indeed tempting to assume that subsequent progressive glomerular lesions develop entirely from such unique areas of lowered resistance and that the tubular lesions evolve secondarily.

While the importance of the changes in the glomerular capillaries should not be underestimated, other possibilities must be considered. For example, similar glomerular tuft changes have also been found in several instances in which clinical recovery has been complete for a year or more, *i.e.*, in a few Evans and Wistar rats that failed to develop chronic recurring nephritis while being fed high or basal protein diets and in those Whelan animals which were maintained on a low protein diet (2). Therefore, it would seem that the pathogenesis of this type of secondary chronic nephritis may depend in part on the response of some other tissue, probably the tubular structures. That tubular lesions may differ in character even in the presence of comparable glomerular scarring has already been observed in Whelan rats with unremitting progressive nephritis (2). In these animals, maintained on a basal protein diet, the distal convoluted tubule was more obviously involved than the proximal, whereas in those Whelan rats fed a high protein diet the converse was true. Furthermore, the recent work of Addis (8) with rats fed high or low protein diets indi-

cates that the amount of protein reabsorbed in the proximal tubule influences the rate of protein excretion in rats with chronic glomerular disease. Nephrectomy of an established chronic glomerular disease in rats does not influence the rate of protein excretion.

Therefore, a decrease in protein excretion in rats subsequent to nephrectomy is not alone on residual glomerular disease. The abnormal glomerular function is a direct result of glomerular disease, and is constantly in a state of progression.

1 Three different types of glomerular disease in the rat: anti-kidney serum disease, gold chloride toxin and most progressive form of the acute nephritis. The anti-kidney serum disease is almost as vulnerable to the effects of nephrectomy as the least affected form of glomerular disease.

2 Both Evans and Whelan have shown that in rats with anti-kidney serum disease, injury, and between the two types of glomerular disease, the anti-kidney serum disease is normal or only slightly affected by nephrectomy. Clinical signs of disease are not observed, only minor scars are seen in the glomeruli.

3 Most of the rats with anti-kidney serum disease progressing chronically have a basal or high protein excretion.

4 Histopathological studies of rats with active glomerular disease followed immediately by nephrectomy after an interval of months in Whelan rats.

5 Some intraglomerular glomerular disease survived acute nephritis in rats. Animals that remained healthy after nephrectomy.

6 The permanent glomerular disease have moderate glomerular disease. Factors other than this recurrent nephritis observed. Unknown factors may produce affecting both glomeruli.



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EXPLANATION OF PLATES

PLATE 14

FIG 1 Kidney of Wistar rat, No 10 in Text-fig 2, which died 10 days after initial injection of anti-Wistar-rat-kidney serum Proteinuria 27 to 51 per cent with numerous casts of all types found on urine analysis Anasarca present at post-mortem examination Glomerular capillary basement membranes are greatly swollen Tubules are slightly dilated with granular material in lumina and with epithelium in various stages of degeneration Mallory-Heidenhain stain $\times 400$

FIG 2 Kidney of Wistar rat, No 8 in Text-fig 2, which was sacrificed $3\frac{1}{2}$ months after injection Average proteinuria per 18 hours was 121 mg during 1st month and 7 mg during final 2 months Irregularly thickened capillary basement membranes and several thick strands of connective tissue are distinguishable in the large tuft Contrast with uniform extensive thickening of capillary basement membranes and lack of connective ingrowth in Fig 1 Mallory-Heidenhain stain $\times 300$

FIG 3 A lower magnification of the slide illustrated in Fig 2 Glomerular changes are less apparent at this power Tubular lesions are minimal $\times 60$

FIG 4 Kidney of male Wistar rat which received a total of 10 cc of anti-Wistar-rat-kidney serum and was sacrificed 3 months later Average proteinuria for entire period was 56 mg per 18 hours Scarring in varying amounts is present in all the glomeruli Nests of atrophic tubules with thickened basement membranes surrounded by fibrous tissue are present but most tubules are slightly dilated and lined by hypertrophic epithelial cells Mallory-Heidenhain stain $\times 85$

FIG 5 Kidney of Wistar rat, No 2 in Text-fig 2, which was sacrificed 3 months after injections of nephrotoxin Average proteinuria for entire period was 73 mg per 18 hours The lesions are similar to those illustrated in Fig 4 Mallory-Heidenhain stain $\times 85$

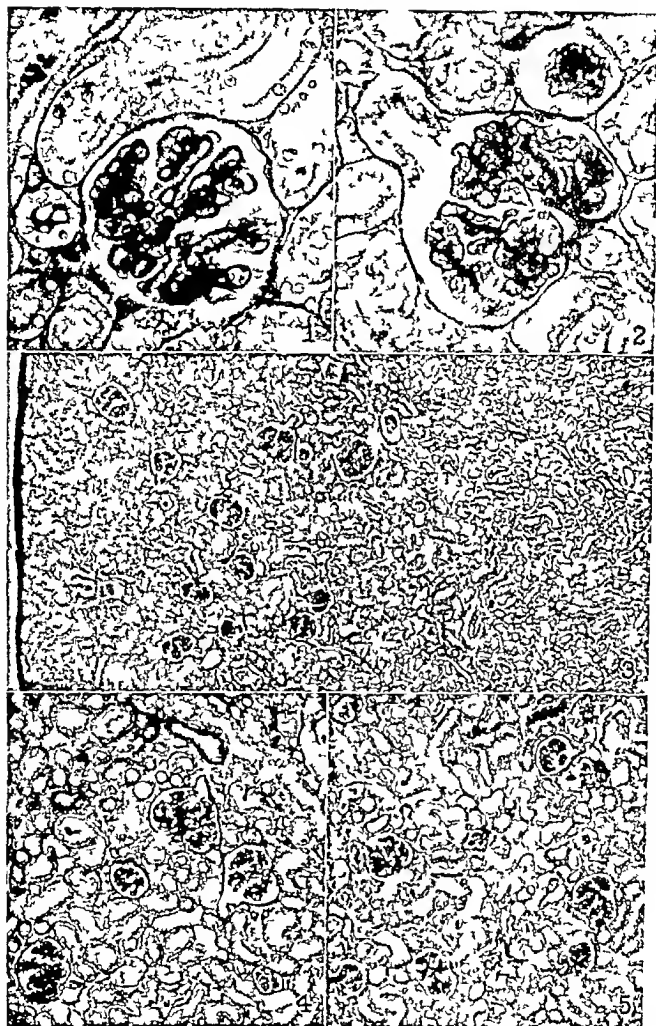


FIG 6 Kidney of female Wistar rat, T-28 Three injections of antikidney serum were administered on successive days Proteinuria of 75 mg per 18 hours occurred during 1st week, 7 mg during 2nd week, and less than 1 mg thereafter Numerous casts were found in 1st week, they declined during next 3 weeks and were absent subsequently Animal was fed a high protein diet and sacrificed 14 months after injections Few abnormalities can be seen at this power Mallory-Heidenhain stain $\times 20$

FIG 7 Kidney of female Wistar rat, T-27 Injection period and 1st week similar to rat T-28 Urinary abnormalities subsided more slowly but by 5th week animal excreted only 4 mg of protein per 18 hours and no casts were found Urine analyses revealed normal findings throughout 2nd and 3rd months Average proteinuria during 4th month was 7.5 mg and rare hyaline casts were seen Slow steady increase in both constituents was as follows 6th month, 30 mg and a few casts, 8th month, 55 mg and numerous hyaline, granular and cellular casts, 10th month, 75 mg and numerous casts, 12th month, 71 mg and numerous casts, 14th month, 130 mg and numerous casts Animal was fed a high protein diet and sacrificed during the 14th month Some distortion of glomerular tufts and scarring of tubules are discernible Several dilated tubules contain large hyaline casts Mallory-Heidenhain stain $\times 20$

FIG 8 Kidney of male Evans rat, T-32 During the 2 weeks following injections of nephrotoxin this animal excreted overnight, 1 to 2 cc of urine which contained between 3 and 4 per cent protein, average 49 mg per 18 hours The disease subsided rapidly, average proteinuria per 18 hours was 8.5 mg for 2nd month and only rare casts were observed Toward end of 3rd month, protein and casts increased Average proteinuria in succeeding months was 4th month, 26 mg, 6th, 40 mg, 8th, 86 mg, 10th, 108 mg, 12th, 101 mg, 14th, 120 mg The number of casts was generally proportional to the degree of proteinuria The rat was maintained on a basal protein diet and sacrificed at the end of the 14th month Histological changes are similar to those shown in Fig 7 Mallory-Heidenhain stain $\times 20$

FIG 9 Kidney of male Whelan rat T-43 1 to 1.5 cc of urine was excreted per 18 hours during the 1st week after the series of injections of nephrotoxin, it contained 2.3 to 5.1 per cent protein (average excretion per 18 hours 41 mg) and very many casts An increase in urine volume with only moderate reduction in proteinuria during the remainder of 1st month raised the average 18 hour excretion for the period to 83 mg 69 mg per 18 hours was the average excretion for the ensuing 7 months with the lowest monthly average, 59 mg, occurring during the 7th month post injection and the highest, 87 mg, occurring during the 8th month The urinary volume gradually increased in the final 3 months of life and terminally reached 10 to 14 cc per overnight period, despite the lower concentration of protein in this dilute urine, the excretion per 18 hours was 120 to 130 mg The animal was fed a high protein diet and sacrificed 11½ months after injection while in the terminal phase of nephritis but before it became moribund

Scarring of glomeruli and tubules is more apparent in this illustration than in Fig 8 Nests of dilated hypertrophic tubules separated by areas of connective tissue replacement are seen Mallory-Heidenhain stain $\times 20$

Figs 10 to 13 A higher magnification of the sections illustrated in Figs 6 to 9, respectively

It is evident from Fig 10, that the glomeruli of the recovered Wistar rat show some residual changes which consist principally of irregular thickening of glomerular capillary basement membranes The process is more severe than that illustrated in Fig 2 The tubular structures have been restored to an essentially normal state $\times 85$

Extensive glomerular and tubular lesions are illustrated in Figs 11 to 13 $\times 85$



QUANTITATIVE CHEMICAL STUDIES ON COMPLEMENT OR ALEXIN

III UPTAKE OF COMPLEMENT NITROGEN UNDER VARYING EXPERIMENTAL CONDITIONS*

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In the first paper of this series (1) it was shown that the combining component or components of guinea pig complement could be estimated in weight units by determination of the quantity of nitrogen added to certain specific precipitates formed in the presence of sufficient active complement. In the second paper (2) the data so obtained were used to trace the relationships of the combining component (C'1) of complement to red cell and hemolysis in hemolysis. It was also shown, by tests at high dilutions with known quantities of antigen, antibody, and complement, that fixation of C'1 in antigen antibody combination could occur in amounts equimolecular with the antibody, or even greater, and that the fixation was relatively little influenced by quantities of antigen above a necessary minimum. An explanation of complement fixation was also given in terms of the union of multivalent antigen with multivalent antibody the same concept which has contributed to the understanding of the precipitin and agglutinin reactions (3-5) and their practical application.

The values given (1) for the average content of C'1 N in guinea pig serum, 0.04 to 0.06 mg. of N per ml., were based upon the addition of C'1 N to antigen antibody precipitates formed in the guinea pig serum, with the combining proportions so chosen that an excess of antibody remained unprecipitated. While the specific precipitates were obtained in finely divided form in this way and could be the more easily washed the presence of excess antibody introduced an uncertainty, since it was possible that complement (C') permitted a given

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amount of antigen to combine with more antibody than in the absence of C', with a resulting increase in nitrogen due to antibody uptake rather than C'1 uptake. It was stated in (1), page 690, that this uncertainty had no basis, and the substantiating experiments are given in the present report. These provided data on the fixation of C'1 N by precipitates formed in guinea pig serum in the region of antigen excess and on the uptake of C'1 N by preformed washed specific precipitates. Experiments are also described on the effect of varying reaction periods and on the relation of the amount of C'1 N bound to the quantity of precipitate used for fixation.

Materials and Methods

Guinea pig serum was used as complement (C'). Inactivated complement (iC') was prepared by heating the serum at 56°C for 50 minutes (thermometer in the serum). Shorter inactivation was effective in abolishing the hemolytic activity of C' but did not always reduce the amount of combining component (C'1) to a minimum. (1) C' and iC' were allowed to stand overnight and were centrifuged again in the cold¹ before using.

Anti egg albumin and antipneumococcus Type III rabbit sera were used with egg albumin (La) and the specific polysaccharide of Type III pneumococcus (S III), respectively, as immune systems. The rabbit antisera were not inactivated, as they were employed at dilutions at which their C'1 N content could not be measured.

Quantitative precipitin estimations were carried out as described in numerous papers (for example, 1, 6) with accurately measured quantities of serum and antigen. Analyses were usually run in triplicate, and the specific precipitates were washed three times instead of twice, as was also done in (1). Comparisons were made of the amount of specific N precipitated in the presence of known volumes of C, iC', and saline. The difference between the values obtained with C' and iC' was considered due to complement combining component nitrogen (C'1 N).

In all except the experiments with preformed specific precipitates antiserum was thoroughly mixed with C' (or iC') and saline before addition of antigen. Hemolytic "units" were measured as in (1).²

EXPERIMENTAL

Experiment 11 Comparison of Reaction Periods of 1, 2, and 3 Hours—Temperature, 19.5°C. Complement "titer," 250 "units." Portion of C' inactivated for 50 minutes at 56°C (iC'). Antipneumococcus Type III rabbit serum B 6, diluted with 5.5 volumes of saline, pooled with corresponding dilution of serum 6 06_{2b}. S III dilution contained 0.04 mg per ml. All determinations in triplicate except blanks. Tube contents were mixed frequently during period of standing.

¹ In an International Equipment Company refrigerated centrifuge.

² The sheep red cells used were kindly furnished by Miss Edna Baker of the Wassermann Laboratory, Presbyterian Hospital.

Hrs. at 19.5 C	1						2		3			
C ml	3.0	3.0					3.0	5.0	3.0	5.0		
iC ml			3.0	3.0		3.0						
Serum dilution ml	1.0			1.0	1.0	1.0	1.0	1.0	1.0	1.0		
S III dilution ml		1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0		
Saline ml	3	3	3	3	5	2	2	2	2	2		
N precipitated mg	0.006	0.004	0.012	0.008	0.456 0.500 0.476	0.518 0.508 0.514	0.588 0.588 0.586	0.630 0.622 0.630	0.590 0.586 0.578	0.626 0.638 0.622	0.582 0.576 0.592	0.622 0.618 0.620
Mean	0.005		0.010			0.513	0.587	0.627	0.585	0.629	0.583	0.620
Subtraction of blank						0.010	0.005	0.008	0.005	0.008	0.005	0.008
Specific N precipitated mg						0.503	0.582	0.619	0.580	0.621	0.578	0.612
Subtraction of iC value							0.503	0.510†	0.503	0.510†	0.503	0.510†
C 1 N precipitated mg							0.079	0.109	0.077	0.111	0.075	0.102
C 1 N per ml. guinea pig serum taken mg							0.026	0.022	0.026	0.022	0.025	0.020

Hemolytic units left in C supernatants <<15

0.005 × 5/3

† In this experiment the saline control values varied unaccountably. It was therefore assumed that the iC N value 0.503 was 0.01 mg greater than the saline series should have been as was frequently found in (1) for 3 ml of iC. For 5 ml 2/3 of 0.01 was added to the iC series value for 3 ml (column 7).

Experiment 12 Fixation of Complement by Specific Precipitate Containing Excess Antigen—Temperature 22 C Complement titer, 285 'units per ml Anti Ea pool C rabbit serum used, diluted with 2 volumes of 0.9 per cent saline. Ea solution in saline contained 0.058 mg Ea N per ml. The supernatant from a mixture of 1.0 ml each of Ea and anti Ea contained a trace of Ea as indicated by a positive test with antiserum and a negative test with Ea. In the experiment itself the contents of the tubes were thoroughly mixed and Ea was added last.

No of tubes	1	1	1	1	1	2	2	3
C ml	3 0	3 0						3 0
iC ml			3 0	3 0			3 0	
Serum dilution ml	1 0		1 0		1 0	1 0	1 0	1 0
Ea dilution ml		1 0		1 0		1 0	1 0	1 0
Saline ml	3	3	3	3	6	5	2	2
N precipitated mg	0 0 10	0 0 08	0 0 16	0 0 18	0 0 02	{ 0 524 0 510	{ 0 526 0 522	{ 0 620 0 620 0 612
Mean	0 0 09		0 0 17		0 0 02	0 517	0 524	0 617
Subtraction of blank.						0 0 02	0 0 17	0 0 09
Specific N precipitated mg						0 515	0 507	0 608
Subtraction of iC series value								0 507
C 1 N precipitated, mg								0 10

Hemolytic units left in C' series supernatants <<25

Experiment 13—Since this experiment was designed to supply comparative data on C'1 N estimations in active guinea pig serum with antigen antibody mixtures in different proportions and with preformed precipitates, no analyses were run in inactivated complement C'1 N was calculated by addition of 0.01 mg of N per 3 ml of C' to the saline control values (*cf* 1) Temperature, 22°C Complement "titer," 285 "units" per ml Rabbit antipneumococcus Type III serum B 6 used, diluted with 3.5 volumes of saline The S III solutions contained 0.04 and 0.12 mg per ml Reaction time, 1 hour and 20 minutes, with frequent mixing

Column	1	2	3	4	5	6	7	8	9	10	11	12
No. of tubes	1	1	1		3	3	3	3	3	3	3*	3*
C' ml			5.0	5.0		3.0	5.0		3.0	5.0	3.0	5.0
Serum dilution ml	1.0	1.0			1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
S III, 0.04 mg per ml ml				1.0	1.0	1.0	1.0				1.0	1.0
S III 0.12 mg per ml ml								1.0	1.0	1.0		
Saline, ml	7	2	2		6	3	1	6	3	1	5	3
N precipitated mg	0.008	0.016	0.014		0.490 0.472 0.496	0.584 0.582 0.578	0.626 0.626 0.622	0.692 0.694 0.698	0.794 0.790 0.794	0.860 0.856 0.842	0.570 0.578 0.574	0.600 0.604 0.616
Mean	0.008		0.015		0.486	0.581	0.625	0.695	0.793	0.853	0.574	0.607
Subtraction of blank					0.008	0.009†	0.015	0.008	0.009†	0.015	0.009†	0.015
Specific N precipitated mg					0.478	0.572	0.610	0.687	0.784	0.838	0.565	0.592
Subtraction of calculated iC' value					0.488	0.495			0.697	0.704	0.488	0.495
C1 N precipitated mg					0.08	0.12			0.09	0.13	0.08	0.10

Hemolytic units left in entire supernatants of first four C series <<16 in each in supernatant from precipitate series with 3 ml C' (column 11), <<16 in supernatant from precipitate series with 5 ml C (last column), <30

In these two sets of tubes S III and antiserum were first mixed allowed to stand 0.5 hour at room temperature centrifuged in the cold washed with 3 ml of chilled saline and again centrifuged The washed precipitates were resuspended in the volumes of saline indicated and the guinea pig serum was then added

† $3/5 \times 0.015$ (column 3)

Duplicate aliquot portions of 8.0 ml of the combined supernatants of the tubes in columns 5, 6, and 7 of the protocol, which originally contained an excess of antibody, were mixed with an additional 0.04 mg of S III and allowed to stand in the cold overnight The resulting precipitates were centrifuged in the cold and analyzed for N in the usual way The quantities found were the same, whether or not complement had originally been present 0.186 mg in the salt supernatants, 0.194 in the 3 ml C' supernatants, and 0.192 in the 5 ml C' supernatants Excess S III was present in the supernatants from these analyses, indicating that antibody precipitation was complete The quantity of antibody N present, calculated from the difference between the specific N values in columns 8 and 5, was 0.21 mg

Experiment 14 Fixation of Complement by Varying Amounts of Specific Precipitate—Temperature, 25°C Complement "titer," 250 "units" 75 ml inactivated at 56°C (thermometer in serum) for 50 minutes A specific precipitate was prepared as follows on the day before use 10 ml of antipneumococcus Type III rabbit serum B 53, containing 3.56 mg of antibody N per ml, were diluted to about 35 ml and mixed with a solution of 2 mg of S III, an amount chosen to leave antibody in excess

After 0.5 hour at 0°C the mixture was centrifuged and the precipitate was washed twice with 35 to 40 ml of chilled saline resuspended in 35 ml of saline containing 1:10,000 merthiolate³ and filtered through a loose cotton plug to remove any large lumps. The N content of the suspension (lot A) was 0.54 mg per ml. Suspensions B and C were prepared from lot A by dilution of 7.5 ml to 10 and 15 ml, respectively, with saline while suspension D was prepared by dilution of 2.5 ml of lot A to 10 ml and suspension E by dilution of 2 ml of lot A to 15 ml. As shown in the protocol, the suspensions were mixed with 7 ml of saline, 7.0 ml of iC', and 7.0 ml of C' and centrifuged after 2 hours at 25° with frequent mixing. Triplicate determinations were run in every instance except with the C' and iC' saline blanks on which single estimations were made.

Suspension 1.5 ml			A	B	C	D	E
Saline ml			7	7	7	7	7
N precipitated mg			$\begin{Bmatrix} 0.720 \\ 0.708 \\ 0.722 \end{Bmatrix}$	$\begin{Bmatrix} 0.540 \\ 0.542 \\ 0.538 \end{Bmatrix}$	$\begin{Bmatrix} 0.366 \\ 0.360 \\ 0.354 \end{Bmatrix}$	$\begin{Bmatrix} 0.178 \\ 0.180 \\ 0.180 \end{Bmatrix}$	$\begin{Bmatrix} 0.104 \\ 0.092 \\ 0.090 \end{Bmatrix}$
Mean			0.717	0.540	0.360	0.179	0.095

Suspensions 1.5 ml			A	C	E	A	B	C	D	E
iC, ml	7.0		7.0	7.0	7.0					
C, ml		7.0				7.0	7.0	7.0	7.0	7.0
Saline, ml	1.5	1.5								
N precipitated mg			$\begin{Bmatrix} 0.764 \\ 0.758 \\ 0.768 \end{Bmatrix}$	$\begin{Bmatrix} 0.382 \\ 0.376 \\ 0.376 \end{Bmatrix}$	$\begin{Bmatrix} 0.108 \\ 0.104 \\ 0.102 \end{Bmatrix}$	$\begin{Bmatrix} 0.874 \\ 0.868 \\ 0.868 \end{Bmatrix}$	$\begin{Bmatrix} 0.660 \\ 0.666 \\ 0.682 \end{Bmatrix}$	$\begin{Bmatrix} 0.464 \\ 0.478 \\ 0.480 \end{Bmatrix}$	$\begin{Bmatrix} 0.274 \\ 0.276 \\ 0.268 \end{Bmatrix}$	$\begin{Bmatrix} 0.176 \\ 0.178 \\ 0.176 \end{Bmatrix}$
Mean			0.763	0.378	0.105	0.870	0.669	0.474	0.273	0.177
Subtraction of blank			0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006
Specific N precipitated mg			0.755	0.370	0.097	0.864	0.663	0.468	0.267	0.171
Subtraction of corresponding iC series value						0.755	0.560*	0.370	0.185†	0.097
C 1 N taken up mg						0.11	0.10	0.10	0.08	0.07
Hemolytic units remaining in supernatant (of 1750 originally)						100	200	280	430	570

* Rough extrapolation between the A and C series values made by addition of 0.02 mg N to the corresponding value in salt series (column 3 first part of protocol)

† Rough extrapolation between the C and E series values made by addition of 0.006 mg N to corresponding value in salt series (column 5 first part of protocol)

Experiment 15 Fixation of Complement by Quantities of S III and Anti-S III in Excess—Temperature about 20°C Complement titer, 250 units 70 ml inactivated as before. Dilutions of rabbit serum pool B 6.56 (3.0 mg of anti S III N per ml) were made with saline as follows: lot A 7.5 ml serum diluted to 20 ml lot B

³ Manufactured by Eli Lilly and Company, Indianapolis, Indiana

5 ml serum to 20 ml, lot C, 2.5 ml serum to 20 ml. Corresponding dilutions of S III, were also made up to contain A, 0.071 mg S III per ml, B, 0.048 mg per ml, and C, 0.024 mg per ml. Blanks, 1 tube each.

Serum and S III dilution used	A					B	C				
C', ml		5.0	5.0					5.0	5.0		
iC', ml				5.0	5.0					5.0	5.0
Saline, ml	6.5	1.5	1.5	1.5	1.5	6.5	6.5	1.5	1.5	1.5	1.5
Serum dilution, ml	1.5	1.5			1.5	1.5	1.5	1.5			1.5
S III dilution, ml			1.5	1.5					1.5	1.5	
N precipitated, mg	0.002	0.018	0.010	0.004	0.014	0.002	0	0.002	0.010	0.016	0.010
Mean blank N, mg		0.014		0.009				0.006		0.013	

Analyses, in triplicate, with 1.5 ml serum dilution and 1.5 ml S III dilution added to each tube.

Serum and S III dilution used	A			B			C		
C', ml			5.0			5.0			5.0
iC', ml		5.0			5.0			5.0	
Saline, ml	5			5			5		
N precipitated, mg	{ 1.134 1.138 1.154	{ 1.166 1.150 1.170	{ 1.264 1.270 1.264	{ 0.756 0.768 0.750	{ 0.762 0.766 0.770	{ 0.874 0.878 0.882	{ 0.374 0.360 0.376	{ 0.382 0.376 0.382	{ 0.482 0.476 0.492
Mean	1.142	1.162	1.266	0.758	0.766	0.878	0.370	0.380	0.483
Subtraction of blank	0.002	0.009	0.014	0.002	0.011*	0.010†	0	0.013	0.006
Specific N precipitated, mg	1.140	1.153	1.252	0.756	0.755	0.868	0.370	0.367	0.477
Subtraction of iC' values			1.153			0.755			0.367
C'1 N precipitated, mg			0.10			0.11			0.11

Hemolytic units left in A, B, C complement supernatants, < 16

* Calculated by interpolation from the A and C series blanks

† " " " " " " " " " " " "

DISCUSSION

The data given in Experiment 11 show that uptake of complement combining component nitrogen (C'1 N) by a specific precipitate was complete in 1 hour at room temperature, and that identical quantities of C'1 N were also added when exposure to C' was continued 1 or 2 hours longer. The apparent solubility effect, previously noted (1), in which less C'1 N per ml was removed from relatively larger quantities of guinea pig serum, was also found to be

uninfluenced by the period of contact of the reactants within the limits given, since in all cases 0.025 to 0.026 mg of C'1 N per ml was taken up from 3 ml portions of C' and 0.020 to 0.022 mg per ml from 5 ml portions.

It is shown in Experiment 12 that specific precipitates formed in active guinea pig serum by addition of a slight excess of egg albumin to rabbit anti egg albumin serum took up more nitrogen than was added in the same quantity of inactivated guinea pig serum. The difference, 0.10 mg, for 3 ml of complement, was of the same magnitude as in previous instances in which an excess of antibody, rather than an excess of antigen, was used (1) (also Experiment 11).

It is also evident from Experiment 13 that identical quantities of C'1 N were removed from active guinea pig serum by sufficient S III rabbit anti S III precipitate whether the specific precipitate was formed in the region of excess antibody or in the region of excess S III. The last portion of the experiment also shows that the supernatants containing excess antibody held the same quantity of antibody when saline inactivated complement, or active complement was present in the original precipitations, showing directly that the combining proportions of S III and antibody are uninfluenced by active complement. In the measurement of C'1 N, therefore, it is immaterial whether a moderate excess of antigen or antibody be used provided the texture of the specific precipitate remains such that efficient washing is possible. In none of the numerous experiments so far recorded, therefore, was the nitrogen uptake in the presence of active complement due to excess antibody and this strengthens the conclusion that the increase observed was actually due to complement combining component.

The data in the last two columns of the protocol of Experiment 13 show that a specific precipitate freshly formed in the region of antibody excess, was almost as efficient in the fixation of C'1 N as were the corresponding quantities of S III and antibody when added separately. The washed specific precipitate removed the same quantity of C'1 N from the 3 ml portions of C' as did S III and anti S III in solution, whereas 0.48 mg of precipitate N was not sufficient to take out all of the C'1 from 5 ml of C'. This is shown not only by the C'1 N values in the protocol, but also by the tests for the number of hemolytic units of C' remaining in the supernatants.

It will be remembered that Goodner and Horsfall (7) showed that precipitation and centrifugation greatly impaired the capacity of specific precipitates to fix complement. The results now reported, which show that under suitable conditions specific precipitates may fix as much C'1 N as antigen and antibody added separately, are not at all in conflict with this conclusion. The specific precipitates used in the present studies were prepared in the region of excess antibody so that they might be resuspended in finely divided form after repeated centrifugation, and pains were taken, by frequent mixing of the tube

contents, to ensure prolonged contact of the precipitates with the C'. The observations of Goodner and Horsfall (7) clearly demonstrated the decisive effect of variation of experimental conditions on the fixation of complement, so that the recording of opposite conclusions under widely different conditions is not only to be expected but may be easily accounted for

In view of the efficient fixation of C'1 by finely divided specific precipitates a study was made in Experiment 14 of the uptake of C'1 N by varying quantities of preformed S III-anti-S III. The relative amounts of C' and specific precipitate were so chosen that excess C' would remain. While it is hoped that additional experiments along these lines will aid in a more complete understanding of the mechanism of complement fixation, these initial data seem significant in their relation to other observations (1, 2). It will be noted that in series E 0.097 mg. of specific N fixed 0.07 mg. of C'1 N, or 72 per cent of its own weight of complement combining component, a result approaching in magnitude the uptake of even more than an equal weight of C'1 N under conditions of greatest sensitivity (2). Double the quantity of specific N removed only a little more C'1 N (0.08 mg.), but it is evident from the remaining data that still larger amounts of specific N removed more C'1 N and also a larger number of units of hemolytic activity. More information will be necessary, however, for the evaluation of the relationship between these two measures of complement activity.

In Experiment 15 identical quantities of C'1 N, within the error of the determinations, were removed from 5 ml. of another pool of guinea pig serum by 0.37, 0.76, and 1.14 mg. of specific N, showing that when sufficient precipitate is present to exhaust the C'1 the amount of C'1 N found is independent of the specific N taken.

The several tests described above serve, then, to confirm the validity of the method for the estimation of complement combining component(s) in weight units, and to extend the experimental basis for its use.

Theoretical implications of the large uptake of C'1 N by preformed specific precipitates will be discussed when more data are available.

SUMMARY

1 Quantitative data are given on the effect of variations in the time of contact and the proportions of the reactants on the quantity of complement combining component nitrogen (C'1 N) found in active guinea pig serum.

2 C'1 N was the same when determined with precipitates containing excess antibody or excess antigen.

3 Finely divided specific precipitates took up the complement combining component (C'1) from subsequently added guinea pig serum almost as well as specific precipitates formed in the presence of complement.

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INFLUENCE OF CHARACTER OF ANTIBODY UPON VELOCITY OF FLOCCULATION

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The velocity of precipitate formation in mixtures of antibody and antigen depends upon a number of factors, including the proportion in which the reagents are mixed, the absolute concentrations of reagents, electrolyte concentrations and probably the amount of certain adjuvants, possibly lipid in nature, which may be present. It is intended to call attention here to the influence of another factor, the character of the antibody.

In a number of antibody antigen systems it has been found that a study of the proportions in which reagents should be mixed to obtain relatively the most rapidly flocculating mixture is of considerable interest and practical value. Two examples which will at once come to mind are the Ramon (32) titration of diphtheria toxin and antitoxin and the Dean and Webb (10) optimal proportions titration. Such methods are also being used more and more in theoretical investigations.

It will be recalled that the Ramon and the Dean and Webb titrations are not done in exactly the same manner. In the Ramon technique, varying amounts of antiserum are added to a constant amount of antigen (toxin) and the most rapidly flocculating mixture observed. This has been called the β procedure by English writers (cf 26). The Dean and Webb technique holds the amount of antiserum constant and varies the amount of antigen (α procedure). In either case the optimal ratio (dilution of antigen/dilution of antiserum) depends within limits of error on the relative, and not the absolute concentrations of the reagents. Thus in the α procedure, if most rapid flocculation occurs in the case of antiserum diluted 1/5 and antigen diluted 1/200 then with antiserum diluted 1/10 the optimal dilution of antigen will be 1/400. This is the origin of the term 'optimum proportions'. This technique has been considerably used in titrating rabbit precipitating sera and their antigens.

Now of course the β or "reverse" procedure could also be applied to these systems. It might not have been too easy to predict *a priori* whether the optimal proportions ratio obtained by application of the two methods to the same system would be the same. A number of experimenters have tried this (11, 27, 34-36), and have found that the two points are not the same. The ratios differ, but by amounts which vary in different systems. An attempt is made to explain this point, by use of a diagram in Marrack's book (26).

The α procedure has seldom been applied to toxin antitoxin titrations, perhaps chiefly because the antiserum almost always contains several times as many units as the toxin, which means that if the toxin is diluted, the antitoxin will have to be diluted even more. The precipitating power of an antitoxin seldom approaches that of a rabbit antiserum against egg albumin, for example, and is less rapid, so it becomes difficult to work with such dilute mixtures. In addition, as is well known, the toxin-antitoxin system differs strikingly from the rabbit antisera, even from rabbit antidiphtheria toxin, in that the range of mixtures which will precipitate is much more narrow, so that it is necessary to have mixtures not too far from the optimum in order to get any precipitation at all.

If the α procedure is applied to the toxin-(horse)antitoxin system, the optimum obtained does not usually differ very much from that obtained by the more usual β procedure (27). This is in contrast to the behavior of most rabbit antisera. It would seem that something remains to be learned by more experiments along these lines.

The present work was undertaken with the aim of exploring a little further the relation between the two sorts of optima, employing a number of antisera, some from rabbits and some from horses, against a variety of antigens. An attempt will be made to present the results somewhat more clearly by use of methods employed in map making.

Technique

The technique employed, a slight modification of that described by Dean and Webb (10), has been used by Dr. Hooker and myself for some time, but never fully described in print. The flocculation was observed in small tubes holding as nearly as possible 1.0 cc., and all of approximately the same internal diameter. An appropriate number of these were placed in a rack kept in a water bath at 37°C. in such a way that each tube was approximately one-third immersed. The approximate position of the optimum was located by a "rough test" in the usual way. Then a set of dilutions of antigen was made in Wassermann tubes, each tube finally containing 0.5 cc. of a mixture 1.5 times as dilute as that in the preceding tube. With a 1.0 cc. pipette 0.5 cc. of the proper dilution of antiserum was added to each Wassermann tube in turn, beginning with the highest dilution. After each addition the contents were mixed by brief shaking, then withdrawn by means of a capillary pipette with a rubber bulb and a long, not too fine tip. The mixture was immediately transferred to one of the smaller tubes. This was placed in the rack and the time recorded. This transfer insures thorough mixing and gives an adequately high column of liquid to allow convection currents to be established to cause mixing to continue, which accelerates the reaction, probably proportionally in all tubes (18). The diluted antiserum was added to the other tubes in turn, and transferred to the small tubes, using the same capillary pipette for each series in which the same antiserum dilution was being used, and going only from the higher to the lower dilutions (from weak to strong antigen). For the next series, a dilution of antiserum 1.5 times as great was used, the range of antigen dilutions extended if necessary, and a fresh capillary pipette used.

The tubes were observed continuously, in daylight or under white fluorescent light.

Cloudiness appears in the tubes which are going to flocculate usually within a few minutes. The time at which particles just visible to the naked eye appear was recorded for each tube. By subtracting the time the tube was filled, the time required for flocculation was found.

The same procedure, with the obvious modifications, can be used for the β method. If a complete checkerboard experiment is being done, it does not matter which procedure is followed, since ultimately all possible mixtures (which will flocculate) of antigen and antibody are tested anyway.

TABLE I

Times of Flocculation in Mixtures Containing 0.5 cc Each of Various Dilutions of Antiserum and Antigen

Serum 1 be No	Antigen tube No																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Minutes																
1	1	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	5	7	11	18			
2	6	1	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$3\frac{1}{2}$	5	7	$12\frac{1}{2}$	20	$30\frac{1}{2}$		
3	$69\frac{1}{2}$	4	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	$4\frac{1}{2}$	$6\frac{1}{2}$	8	11	23	$31\frac{1}{2}$		
4		>90	11	$1\frac{1}{2}$	1	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	$2\frac{1}{2}$	4	$6\frac{1}{2}$	9	15	20		
5			>90	$31\frac{1}{2}$	3	$1\frac{1}{2}$	$1\frac{1}{2}$	$1\frac{1}{2}$	2	$3\frac{1}{2}$	$4\frac{1}{2}$	$6\frac{1}{2}$	10	$14\frac{1}{2}$	24	$34\frac{1}{2}$	50
6				>90	14	3	2	$2\frac{1}{2}$	$2\frac{1}{2}$	$4\frac{1}{2}$	6	8	12	$16\frac{1}{2}$	28	40	60
7					>90	10	5	4	4	4	6	8	10	17	29	$40\frac{1}{2}$	51
8					>90	$41\frac{1}{2}$	17	$6\frac{1}{2}$	6	6	$6\frac{1}{2}$	9	15	20	27	$41\frac{1}{2}$	67
9						>90	$54\frac{1}{2}$	16	9	9	9	11	18	25	38	54	$69\frac{1}{2}$
10							>90	65	$15\frac{1}{2}$	12	12	13	16	26	33	46	$64\frac{1}{2}$
11								>90	$75\frac{1}{2}$	28	20	20	23	33	43	59	
12										>90	57	32	35	43	54		

The reagents were a rabbit antiserum (No 113) against *Limulus* hemocyanin and its antigen. The antigen and antiserum dilutions were successively 1.5 times greater in each tube, reading from left to right for the antigen and from top to bottom for the antiserum. The serum in the first tube was diluted 1:2, the antigen 1:5 (for absolute concentrations, see Table II).

By carrying this procedure as far as the point at which the most rapid tube in a series took $1\frac{1}{2}$ hours or over, the whole range of possible flocculating mixtures was covered, and a table of times of flocculation obtained, one entry for each combination of antibody dilution and antigen dilution. An example is shown in Table I.

Successive dilutions in steps of 1:1.5 were selected as not being too laborious and giving sufficiently precise information as to the relation between times of flocculation and the proportion in which reagents were mixed.

RESULTS

The results of such an experiment as that shown in Table I can be plotted in a number of ways. If we plot the times of flocculation against the dilution of

antigen used, for each serum dilution, we obtain the picture seen in Fig 1, which illustrates the principle of the α procedure. It will be noted that in all cases except those in which the reagents were too concentrated to allow of proper performance of the test, a well defined minimum is seen in the time curve, corresponding to the point of optimal proportions. The time may be approximately constant in several tubes near this point, owing to the flatness of the curve. If we select the middle tube in such instances as the optimal tube, it will be seen from Table I how the optimal points lie approximately on a straight line, which passes diagonally from upper left to lower right, showing the ratio to be nearly independent of the absolute concentrations.

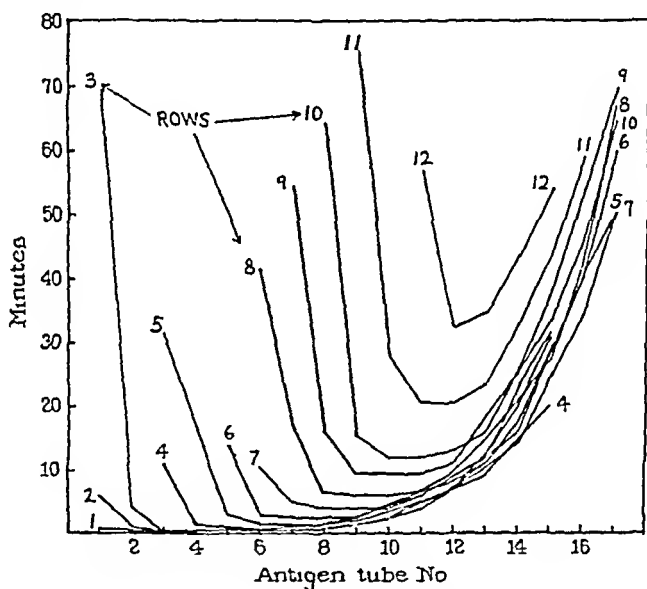


FIG 1 Times of flocculation of serum dilutions, held constant, when mixed with various dilutions of antigen

Fig 2 shows the results of plotting the time against the serum dilution, antigen being held constant. This corresponds to the β procedure of the British writers. It will be noted that although there is some suggestion of a minimum, it is not nearly so well defined as in Fig 1. We may conclude that in this case the β optimum is not so clear as the more usual α optimum. Curves similar to those of Figs 1 and 2 have been presented by Miles (27), based on experiments with agglutinating sera. Compare also Brown (7).

In passing attention may be called to the regularity of the individual curves in these graphs and the systematic way in which each differs from the one next to it, somewhat suggesting a mathematical family of curves, all having the same formula, but different values of the parameters.

Considerations of space would prevent giving similar graphs of all the present data, even if that seemed the best method of presentation. Fortunately a more compact method is available. Mathematically, the set of data shown in Table I defines a surface, where time is the third dimension, and antigen dilution and serum dilution are the two independent variables. (Note that we are plotting these on a logarithmic scale; this is the result of giving simply the tube numbers instead of actual concentrations, but is in any case necessary in order to achieve sufficient compactness.) This surface could be modeled in three dimensions,

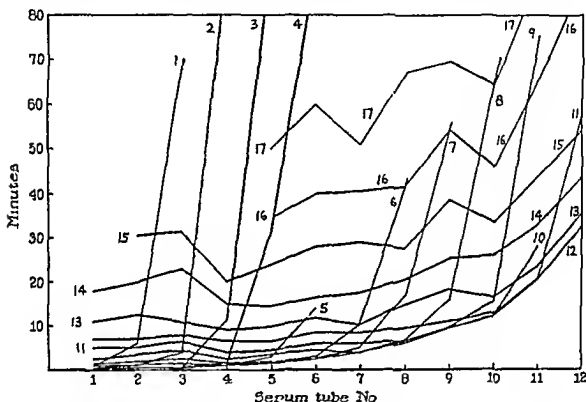


FIG 2 Times of flocculation of various antigen dilutions held constant when mixed with various dilutions of antiserum

and the model would summarize the flocculative behavior of the system. Such a model is not easy to show in two dimensions, but the essential characteristics can be shown by making use of the same conventions used in topographic maps. Lines can be drawn through points of equal time at appropriate intervals, and the position and distance apart of these contour lines (isochrones) serves to characterize the surface. This can be done without actually constructing the surface at all by simply drawing such lines through a table such as Table I having constructed it in such a way that the scale in each direction is the same, and finally eliminating everything but these contours and the coordinates.

Fig 3 shows the results of this procedure applied to the data of Table I. The contour lines cannot be drawn at equal intervals; therefore the intervals have been successively doubled. That is, contours have been drawn for times of 1, 2, 4, 8, 16, 32 and 64 minutes. This happens to produce a set of lines which

are nearly equally spaced in the graph, showing that the slope of the surface increases rapidly in steepness as the dilutions of antibody and antigen are increased. The line of dashes connects the minima obtained by the α procedure, while the line of dots connects those of the β procedure. For a constant "optimum" to be obtained by either method, the corresponding line should be straight, with a slope of 45° .

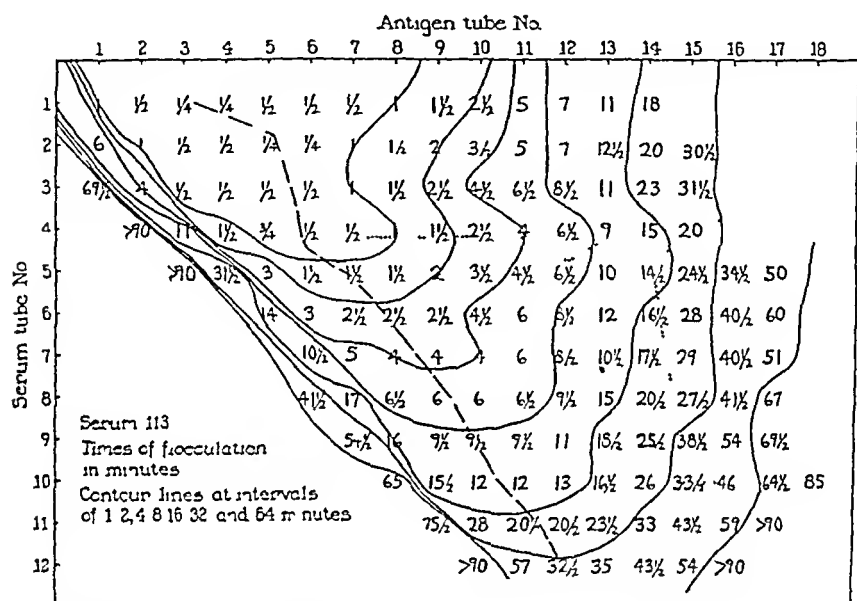


FIG 3 Table of times of flocculation of mixtures of various dilutions of antigen (*Limulus*) (top), and various dilutions of antiserum 113 (left), showing method of drawing lines of approximately equal times. Numbers indicate rows or columns respectively, the dilution of serum in the top row was 1/2, the antigen in column 1 was diluted 1/5 (see Table II)

This technique enables us to present simultaneously the data from a number of "checkerboard" experiments such as that of Table I. Such experiments were carried out with rabbit antisera anti-*Limulus* hemocyanin, 110, 113, 116, 863, 864, anti-cancer hemocyanin, 784, 785, 944, anti-*Homarus* hemocyanin, 778, 9, anti-*Busycou* hemocyanin, 232, anti-ovalbumin, 125, 129, 848, 862, 865, anti-diphtheria toxin, R-T, obtained from Dr Pappenheimer, and horse antisera anti-ovalbumin (H-Ov) and antitoxin (H-T), from Dr Pappenheimer, and anti-*Busycou* hemocyanin (H-633), obtained through the courtesy of the Lederle Laboratories.

The antigens used in these experiments had been prepared as pure as pos-

sible,—by repeated crystallization in the case of ovalbumin, by repeated isoelectric precipitation in the case of the hemocyanins

The exact concentrations of the antigens used, and the antibody content of the antisera, do not bear significantly on the arguments to be presented here

TABLE II
*Optimal Dilutions of Sera and Antigens at Most Concentrated Optimum**

Serum No	Antigen	Concentration of antigen <i>mg N/cc</i>	Lowest optimal dilution	
			Serum	Antigen
	<i>Lamulus</i>			
110	Hemocyanin	5 45	1 2	1 11
110-2				1 3 3
113		'		1 17
116				1 25
863			"	1 15
864				1 47
	<i>Cancer</i>			
784	Hemocyanin	2 50		1 15
784,5				1 10
944		'		1 15
	<i>Homarus</i>			
778 9	Hemocyanin	5 41		1 76
	<i>Ovalbumin</i>			
125		5 60		1 456
129				1 203
848			'	1 304
862				
865				
H-Ov			Undiluted	1 3000
R T	Diphtheria toxin		'	1 3 4
H T	'		1 5 3	Undiluted
	<i>Busyon</i>			
232	Hemocyanin	7 51	Undiluted	1 17
H-633				1 2 25

* Point represented by a cross in Figs. 4 a and 4 b

However they may be obtained from Table II, the former directly, the latter by estimation (2) from the optimal ratios and the published values for the ratio of antibody to antigen in optimal precipitates from the various systems (18, 19 20, 22 25, 28) The horse antitoxin had a strength of 400 units per cc The combined results are presented in Fig 4

It is at once evident that the direction of the dotted or β line, is in general

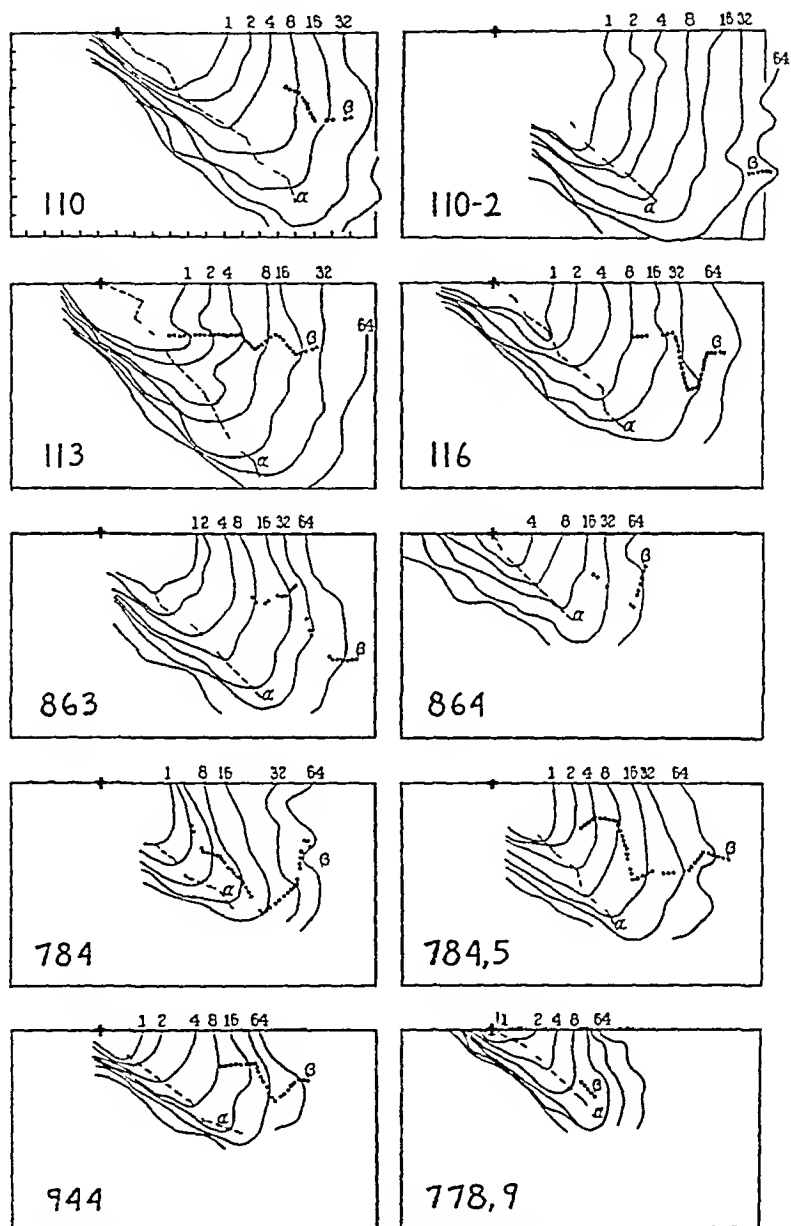


FIG 4a

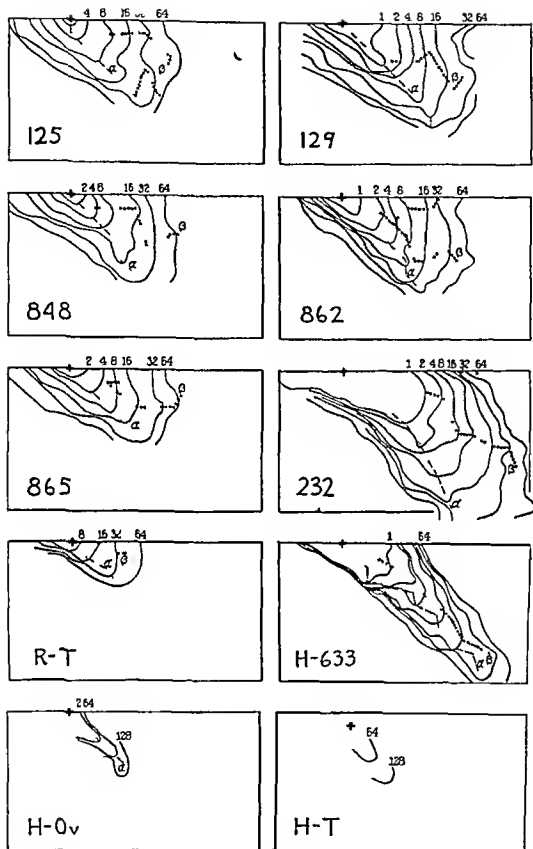


FIG 4b

FIG 4 Contour lines of equal times (isochrones) of flocculation in mixtures of various antigens and antibodies. Scale of dilution, successively 1.5 times greater shown only in graph for serum 110. Numbers at top indicate times α indicates line connecting Dean and Webb optima, β line connecting Ramon optima. The cross indicates the position of the α optimum with most concentrated serum (lowest point on the time surface, see Table II)

much less regular than that of the α line. However, for the sake of fairness, the β line has been indicated in all cases where there was even a suggestion of it, although it was clear that in some cases it was too irregular and unsystematic to have any significance. Note how in a number of cases it has a slope different from 45° , indicating that the β optimum was not constant for different dilutions of antigen. By inspection of Fig 4, it is seen that the sera tested may be divided roughly into two classes, which we may designate as R and H (the reason for this choice of letters will become clear as we proceed)

Definitions

In one class, which we shall call the R class, there is little tendency for well marked consistent minima to be found when the β procedure is followed. If we follow any one isochrone in the graph of such a system in Fig 4 (corresponding to some definite time of flocculation) from left to right, we find that it tends to pass through a minimum where it is concave upward, then to rise pretty much vertically (*i.e.* following some constant antigen dilution) to the upper limits of the graph. Essentially such behavior is found with *rabbit* sera 110, 110-2, 113, 116, 864, 129. Miles (27) has described rabbit agglutinating sera which also behaved in this way.

In systems of the H class, which is most strikingly represented, even though two of the sera are weak, by the three *horse* antiprotein sera (H-633, H-Ov, and H-T), the contour line passes through a minimum as before, then curves over to the left, so as to create another minimum under the conditions of constant antigen. This can be seen by turning Fig 4 clockwise through 90° , so that the right hand side as viewed in the usual way becomes the bottom. In these sera, apparently, combination of more than a certain amount of antibody with a given amount of antigen does not leave the rate of flocculation at its maximum, but actually diminishes it. As already mentioned, this behavior is most striking in the case of the horse antiprotein sera, but to a less extent it is observed also with the rabbit sera 863, 784, 5, 944, 848, 232.

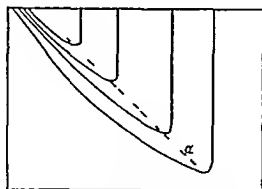
Duncan (11), Miles (27), and Taylor (34) have reported experiments in which this behavior of certain rabbit sera is even clearer.

Horse antitoxin of course furnishes the classical example of what is meant here by an H serum, in the results presented (H-T) this is somewhat obscured by the fact that horse antitoxin is so weak in comparison with the other sera that, on the scale necessary to include the full range of all the sera, the shape of the time surface is not properly shown for this system. An experiment done with smaller steps between successive dilutions would show a picture similar to that given for serum H-633.

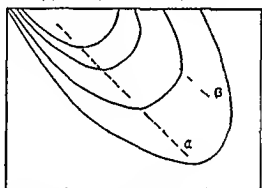
It will perhaps make the distinction between these different types of antisera clearer if we present a set of ideal contour lines illustrating them, in which the non-essential irregularities have been smoothed out. This is done in Fig 5,

which shows the ideal behavior of the R type, the H type, and the extreme H type as typified by dipbtheria antitoxin

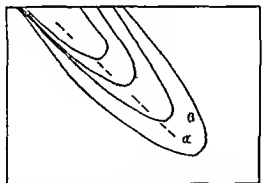
It will be seen that in the R type only one optimum, that obtained by the α procedure, is possible. The β procedure, where increasing amounts of



(A) R TYPE (ONE OPTIMUM)



(B) H TYPE (TWO OPTIMA)



(C) EXTREME H TYPE (OPTIMA CLOSE TOGETHER)

FIG. 5. Ideal Isochrones for antisera of various types (see text)

antibody are added to constant amounts of antigen, will give, for any given amount of antigen more and more rapid flocculation, until the rate reaches a maximum, after which further addition of serum will not influence the rate of flocculation (the volume being kept constant)

In sera of the H type two optima are possible, depending on the procedure, and it is clear from Fig. 5 that these two optima can never actually coincide. With sera of the extreme H type, however, with which the change in slope of the curves is extremely rapid near this point, the difference between the two

optima will be slight. If the isochrones are mathematically similar (the same shape) it can be seen how the quotient of the α and β ratios would be constant for a serum of the H type, as has been reported (11, 34)

DISCUSSION

Considering the difference between the horse antisera and the rabbit antisera, when directed against the same antigens (Nos 129 and H-Ov, R-T, 784,5, and H-633), and the differences in the behavior of different rabbit antisera against the same antigens, it would seem that the flocculation behavior must depend in part on the physical and chemical characteristics of the antibody. Otherwise it is difficult to understand how some sera can belong to the R (one optimum) type, while others directed against the same antigen can belong to the H (two different optima) type. There are perhaps two ways in which antibodies might differ so as to cause these differences in behavior.

It is known that the horse antiprotein antibodies thus far studied differ from the corresponding rabbit antibodies in being more soluble, i.e., a higher concentration of sodium or ammonium sulfate is required to precipitate them. This suggests that the solubility characteristics of the antibodies have a bearing on our problem. If an antibody is essentially rather soluble, it can be understood that combination of more than the optimal amount of it with a given amount of antigen would produce in the region of antibody excess compounds more soluble than those formed at the optimum. The compounds with the larger amounts of antibody, might have predominantly the solubility characteristics of the antibody, thus accounting for the increased solubility and lowered rate of flocculation. Or the surface properties might pass at or near the optimum through a minimum in respect to some other property, such as electric charge.

There is now a good deal of evidence that the primary stage of serological reactions is extremely rapid (3, 5, 12, 13, 16), combination probably being substantially complete in a matter of seconds. Since flocculation is relatively slow, requiring in the case of some antivenoms a number of hours, we may regard flocculation as essentially an expression of the secondary combination of primary aggregates consisting essentially of antigen combined with more or less antibody.

If secondary combination is regarded as more or less non-specific, as in the Bordet theory (1), we may suppose that the rate of flocculation would depend upon, among other factors, the degree of the hydrophilic or hydrophobic character of the antibody. We might suppose that the reason mixtures flocculate more and more rapidly, up to a certain point, as more antibody is added, is that polar groups on the antibody and antigen molecules neutralize each other or are covered up mechanically, making the primary aggregates less and less hydrophilic, and thus more inclined to flocculate. If the antibody

were hydrophilic addition of still more would be expected to produce compounds having again a more hydrophilic character, due to the greater proportion of antibody in them, and thus the tendency to flocculation would have passed through a maximum. The β optimum with sera of the H type would thus be explained. With less hydrophilic antibody, however, the rate of flocculation would be increased up to a certain point as before but the presence of excess antibody would have little or no effect in again increasing the hydrophilic character of the aggregates, particularly as some increase in the number of hydrophilic groups would be required to keep these larger aggregates in solution anyway. This would account for the behavior of the R type of serum. A similar suggestion was made by Brown (7).

In this connection it may be worth while to recall the work of von Smoluchowski (33), who proposed a theory of the velocity of colloidal flocculation, which accounts very well for the results obtained with a number of inorganic sols. His theory, based only on the assumptions of the kinetic theory, assumes that the velocity depends on two different factors,—the number of centers of aggregation initially, and the fraction of collisions between these which result in union. In the β procedure (constant antigen) the number of centers of aggregation is the same in each tube, for we may suppose it substantially equal to the number of antigen molecules present (18), but it might well be supposed that the fraction of collisions resulting in union depends upon the amount of antibody combined with a molecule of antigen. This should be so even if we think of the second stage as being "specific" (14, 26). For here too it is easy to imagine that after the first stage of combination the probability that any two of these primary aggregates will combine on colliding is at first increased, by higher antibody antigen ratio, roughly in proportion to the amount of antibody combined. For, up to a point, the more antibody there is on each aggregate, the greater the chance that one of the free combining groups of an antibody molecule will come sufficiently close to a free combining group of an antigen molecule forming part of another aggregate to unite with it. But after a certain point, the presence of more combined antibody will interfere with aggregation as each aggregate will have proportionally less antigen surface exposed, so that fewer antigen combining groups will be available to effect combination.

The chief difficulty with this second way of looking at the problem is that on such a basis it would appear that all antisera should belong to the H type, as this argument should apply equally to all systems. Pappenheimer, Lundgren and Williams (29) have proposed an explanation of the inhibition zone observed with antibody excess in the toxin antitoxin system which might partly meet this difficulty. They suggest that the combining groups (assumed to be two per molecule) in horse antitoxin are concentrated at one end of the molecule, whereas in rabbit antihodies, including rabbit antitoxin, they are more

evenly distributed. Then it is supposed that the inhibiting effect of antibody excess on flocculation in the case of horse antitoxin is due to the fact that these combining groups of the antibody interfere with each other when more than a certain amount of antibody is combined with a molecule of antigen. In the rabbit system, the combining groups, being further apart, would interfere less, or not at all, with each other, allowing aggregation to proceed. If this were the correct general explanation, we should be forced to assume that practically all degrees of asymmetry in the distribution of the combining groups on antibody molecules could exist, to account for the variation observed in sera from the extreme H type of horse antitoxin to the R types of certain rabbit antibodies. This is perhaps a difficulty, and there is the additional objection that there is thus far no evidence that most of the antibodies that we have studied have more than one combining group per molecule, and the proposed explanation could not apply to a univalent antibody. It does not seem possible at present to decide definitely between the two possible explanations, but the writer still prefers that based on differences in the hydrophilic character of the antibodies, as being on the whole more plausible. At any rate it would seem that observations on the relation of the different precipitation optima can be accounted for, in theory at least, without making any use of the assumptions of the lattice theory.

The optimum observed in flocculation by the α procedure is not difficult to understand. Here the amount of antibody is constant, and the antigen is varied. In the mixtures containing the smaller amounts of antigen, there are not enough centers of aggregation to give the most rapid flocculation, although, in the R type of serum, these centers are maximally coated antigen molecules having about the maximum tendency to aggregation. In mixtures with more antigen, the number of centers is greater, but at the same time the amount of antibody available for each antigen molecule is diminished, and the primary centers resulting are less hydrophobic (more soluble). Finally a point is reached where the diminished tendency of the primary aggregates to combine balances the increase in rate caused by their larger number. As more antigen is added, the tendency to combine is decreased sufficiently to produce the zone of partial, and ultimately of complete, inhibition.

As before, we could also interpret this in terms of the "specific" lattice theory, with about the same degree of plausibility.

Relation of Flocculation Optima to Neutralization

Dean and Webb (10) found that at the optimum obtained by their (α) procedure, in the case of the system they worked with (rabbit-antihorse-serum and horse serum), there were no more than traces of antibody and antigen in the supernatant. Similar results have been obtained with ovalbumin, α - γ -protein, yeast gum, and pneumococcus polysaccharide Type I (9, 11, 21, 36,

and references in 26) This suggested that the flocculation optimum had in addition to its practical utility some theoretical significance

However Burnet (8) found most rapid flocculation of staphylococcal toxin and antitoxin in mixtures containing some excess of antibody and a number of others have obtained similar results Malkiel and Boyd (25) found that in two hemocyanin antihemocyanin systems most rapid flocculation occurred in mixtures in which antigen was in excess

In the Ramon titration (β procedure), the most rapidly flocculating mixture may be neutral, although it is sometimes under, more often over neutralized Pappenheimer and Robinsoo (30) even by the sensitive rabbit intracutaneous test, failed to detect any appreciable toxin or antitoxin in the supernatant from such mixtures Duncan (11) and Taylor, Adair, and Adair (36) found instances in which the Ramon optimum was in the region of antibody excess

The fact that the β optimum corresponds at least in some cases to a point in the equivalence zone may be accidental, but it is more likely that it is due to the fact that in both cases the deciding factor is approximately complete coverage of the surface of the antigen by antibody This is conceivable on the basis of either the older or the newer theories of reaction mechanism, as can be seen by the remarks of Pauling (31) The studies of Boyd and Hooker (4, 6) on the ratio of antibody to antigen in precipitates suggest that antibody covers more surface than if it behaved as a sphere, and purely chemical and physical evidence indicates that antibody molecules are elongated ellipsoids In the case of ovalbumin the antigen surface is calculated to be completely covered by about three molecules of antibody The studies of Heidelberger (14) and collaborators suggest that the compound formed in this system at the antibody excess end of the equivalence zone is mostly AG (in which A represents antibody, and G antigen) This may mean that coverage of about two-thirds of the surface suffices to bring about maximal alteration in surface properties

According to Pappenheimer, Luodgren, and Williams (29), at the β optimum two molecules of antitoxin are combined with one of toxin The formula of Boyd and Hooker would lead to the conclusion that four antibody molecules are required to cover the surface of a molecule of toxin, if this is correct, the surface is in this case only half covered Since horse antitoxin is strongly of the H type, this might be the point at which the hydrophilic properties of the toxic antitoxin compound were at a minimum The observation that such mixtures are nevertheless often neutral could be accounted for if either the number of toxic groups on the toxin molecule is limited or neutralization is due, at least in part, simply to the low solubility and decreased mobility of the compound

One would expect to find that in general the α optimum corresponded to a point in the region of slight antigen excess where the two opposing tendencies

mentioned above could be expected to balance. This was found to be the case for two hemocyanins studied by Malkiel and Boyd (25), but with a number of systems the α optimum, as mentioned above, has been found to correspond to a point in the equivalence zone, and in one case to a point in the region of antibody excess. It may be that the slowing effect of diminished antibody/antigen ratio asserts itself very soon after complete surface covering has been effected, and that antibody combining with a molecule of antigen already completely covered is held somewhat more loosely. If the process of primary combination follows a course mathematically equivalent to an adsorption reaction, as is not improbable, this would be expected.

The statement that the process resembles an adsorption reaction is not intended to imply anything more than that antigen possesses a number of combining groups per molecule, and any mathematical treatment of such a reaction will give results agreeing with one of the various adsorption equations (17, 24).

In a number of antibody-antigen systems it is found that "equivalent" mixtures (mixtures which leave neither antibody nor antigen, or minimal traces of both, in the supernatant) may be obtained over a zone in which the proportions of reagents varies slightly. This has been termed the equivalence zone (15). Heidelberger and Kendall (15) have suggested that "The breadth of the zone in some instances may explain the failure of the 'optimal proportions' method to yield the same end-point when the antigen is diluted as when antibody is diluted, since the equivalence zone would be approached from a different side in each instance." It is clear from the data presented here that this is not the explanation of the difference between the two optima, especially since usually one or the other, sometimes both optimal points fall outside the equivalence zone.

It is seen that the difference between the R and H types of antibody can only be accounted for on the assumption that the chemical and physical properties, or both, of the antibodies have a bearing on their flocculative behavior. This statement would seem to hold, whether the important differences prove to be differences in solubility, or as Pappenheimer, Lundgren, and Williams suggest, variations in the spatial arrangement, or in the extent to which the different combining groups on any antibody molecule affect each other (23). The addition of such assumptions to the lattice theory as it was originally formulated seems now to be admitted to be necessary even by its originators. We can no longer expect to account for the whole course of the precipitin reaction without taking some account of the characteristics of the antibody molecule itself.

I am indebted to Dr. Pappenheimer for sending our laboratory some of his horse anti-ovalbumin serum, and to Dr. Pappenheimer and Dr. Robinson for the gift of

diphtheria toxin, rabbit antitoxin, and horse antitoxin Credit is due to my assistant Mrs Manya K Grossman, for able assistance with the technical work

SUMMARY

Results of a thorough study of the rates of flocculation of 20 antisera when mixed with their antigens in all proportions are presented The relation between the α (constant antibody) and β (constant antigen) optima is discussed It is suggested that most of the antisera examined can be classified into two main types, one of which, the H type gives an optimum by both the α and β procedures, whereas the R type gives an optimum only by the former technique It appears that these differences can only be accounted for by the influence of differences in the physical and chemical properties of the various antibodies

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EXPERIMENTAL GONOCOCCUS INFECTION OF THE CHICK EMBRYO

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PLATES 16 AND 17

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The chick embryo in recent years has been infected with a variety of bacteria, and the reactions to such infection have been found to parallel closely in their basic features those of the respective natural diseases in man (1)

The chorio-allantoic membrane of embryo chicks was first infected with gonococci by Morrow and Berry (2) in 1938, and they were able to cure the infection with sulfanilamide Hill and Pitts also (3) established 15 strains of these microorganisms on the membrane Since then the membranal infection has been used more extensively for the study of the relative efficacy of sulfanilamide and its derivatives (4) However, the reaction of the chick embryo to the gonococcus and the adjustment of it to continued growth in this environment has not been carefully studied

We have attempted to do so by study of both membranal inoculation of 10 day embryos and intra amniotic injection of 14 to 15 day embryos

Method

The course of an experimental infection may be followed by making a series of samples from one living host, or by the infection of a number of hosts and the subsequent fixation and sectioning of certain ones Both of these methods were followed in the study of the reaction of the chick embryo to nine different strains of gonococci

Eight strains of *Neisseria gonorrhoeae* were isolated from typical cases of acute gonorrheal urethritis at Marine Hospital, Baltimore, and Vanderbilt Hospital Nashville They were identified by the following characters small smooth glistening colonies on heated blood agar positive oxydase reaction Gram negative staining, and typical biscuit shaped diplococci One strain was furnished by the American Type Culture Association

The membranes of 10-day old chick embryos were exposed according to Good pasture and Budding's method (5) and infected either by means of a platinum wire loop from a 24 hour heated blood agar plate or a drop of saline suspension of gonococci of about 100 000 per cc Cover slips were placed over the opening and sealed with vaseline or the egg shell flap was replaced and sealed with paraffin The

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infected eggs were reincubated at 36°. Smears and occasionally cultures were made with a platinum wire loop. Gram stains were done on all smears.

14- to 15-day old chicks were inoculated intra-amniotically with 0.1 cc. of standard saline suspension of gonococci from a 24 to 36 hour heated blood agar plate. The technique has been described by Polk, Buddingh, and Goodpasture (6).

Histological sections were made of representative embryos at various stages of infection. After the amniotic fluid had been smeared and cultured the embryo was fixed in Zenker's fluid with 10 per cent acetic acid. Paraffin blocks were made of the head, thorax, and abdomen, and the sections were stained routinely with hematoxylin and eosin. Those sections which showed histological evidence of infection were stained for bacteria with Wright's stain.

General Findings

Gross Appearance of Membranal Infection—The 24 hour infected membrane may show little or no clouding, with only a dull sheen everywhere, or the leucocytic response may be more localized, and small pockets of pus may collect in depressions of the membranes. The latter type is more apt to occur when the membrane is infected by means of a loop, while infection with a drop of saline suspension commonly yields a more diffuse reaction. In the more heavily infected embryo small hemorrhages may occur throughout and clouding is particularly concentrated along the course of the large vessels.

Later, one of three things may happen. (1) The infection may completely disappear, leaving a normal appearing membrane. (2) A localized ulcer with ragged edges and containing pus in its center may form. (3) Finally, the infection may overwhelm the embryo, the vessels becoming thrombosed and the membrane almost devoid of circulating blood.

Smear of Membrane—Since the gonococci rarely penetrate deeply into the chorio-allantoic membrane of 10 day chicks, a day-to-day study of smears from the surface of the membrane is a particularly useful method of following the infection. This is entirely analogous to a study of urethral smears from human cases of gonorrhea.

A leucocytic response to the bacteria occurs within 4 hours in some cases and is always present within 12. It varies in intensity, as does the number of organisms. Although some phagocytosis always appears, the majority of organisms are usually not phagocytosed. More extensive phagocytosis occurs in the "localized" infections where almost all the gonococci may be intracellular.

Effect of Immune Sera on Phagocytosis—Chicken immune serum was obtained by the injection of saline suspensions from 48 hour blood plate cultures into 10 adult chickens twice weekly for 2½ months. These sera agglutinated in 1:160 dilution. Introduction of two drops of serum onto the surface of membranes infected 4 to 24 hours previously failed to increase phagocytosis above that of controls in two experiments. Three controls and three experi-

mental embryos were used in each experiment. Clumping of the gonococci could however be seen in the smears for several hours after addition of serum.

Progress of the Infection—The embryo may die within 2 or 3 days, or may localize and eventually throw off the infection. When the majority of organisms are phagocytosed the chances of recovery are very great, and chicks usually hatch out normally. Avery (7) found that 10 day embryos inoculated with one strain of *N. gonorrhoeae* on the membrane, occasionally yielded on hatching a chick from which the organisms could be recovered by culture of the blood during the first 1 or 2 days.

Distribution of Infection—The extent of the infection and the deep reaction of the tissues can be studied in histological sections. These were made at 12 hours and at daily intervals up to 5 days. The membrane at 24 hours is only slightly thickened; there is a slight exudate consisting of polymorphonuclears, a few mononuclears, and occasionally a little superficial hemorrhage (Fig. 1). The ectodermal layer may be reduplicated at scattered points and occasionally ulcerated.

By the 2nd day the ulceration of the ectoderm has become more marked and the ectoderm itself has thickened. The exudate is heavier, more plastic (Fig. 2), and contains clumps of organisms, some of them phagocytosed by polymorphonuclears. Scattered focal hemorrhages occur deeper in the edematous membrane. If the membrane has been inoculated with a loop, the necrosis and reaction may be concentrated, so that a crater-like ulcer is formed (Fig. 3) by the central destruction of ectoderm and consequent exposure of the mesoderm. It is filled with cell detritus, polymorphonuclears, and scattered organisms. The edges are formed by proliferating ectoderm. A rare polymorphonuclear cell with organisms may be found in the edematous mesoderm. At this stage the embryo itself often succumbs, apparently due to toxic products from the membranous infection accounting for multiple scattered small hemorrhages observed throughout the various tissues and organs. Bacteria are not demonstrable in the embryo.

By the 3rd day and thereafter the whole structure of the membrane may be changed. Islands of ectoderm may have been broken off and caught in the thickened, inflamed mesoderm. The destruction of cells is less extensive, but hemorrhages in the mesoderm are more common. Most remarkable in all of these sections is the absence of the gonococci in the deeper portions of the membrane. Apparently the mesodermal reaction is due to some diffusible product of the bacteria rather than to the presence of the bacteria themselves.

Extent and Distribution of Infection in 14 to 15 Day Embryos—Intra-embryonic injection of 14 to 15 day embryos exposes a variety of tissues and organs to infection. It has been particularly successful in the study of the meningococcus, whereby septicemia and meningitis were produced (Buddingh and Polk (8)). Four strains of gonococci were studied in this way.

Three of the strains were isolated at the Vanderbilt Hospital and the fourth was furnished by the American Type Culture Association

As a rule living infected embryos were killed and fixed for histological study 24 and 48 hours and occasionally 72 hours after inoculation. About equal numbers of each strain were studied. Cultures of amniotic fluid and heart blood were taken at this time from 28 of 41 embryos studied in this manner.

The results are summarized in Table I

All of the amniotic cultures were strongly positive. The positive blood cultures showed 6 to 8 colonies per drop.

Of the 41 embryos studied histologically only 14 showed a reaction to infection. Pneumonitis and bronchitis (Fig 4) were most common but in no case was there complete occlusion of the bronchi by exudate nor was a widespread pneumonitis present (Fig 8). Sinusitis and infection of the thoracic and ab-

TABLE I
Heart's Blood Cultures

Strain	No. of cultures	No. positive
L B	4	0
L B	8	3
After 15 chorio-allantoic membrane passages		
A W	10	0
E M	6	0

dominal air sacs were also common (Fig 5). The latter presumably represented a direct extension from the pneumonitis.

In only one embryo was a purulent meningitis observed (Fig 7). This embryo had a positive blood culture. The meninges were thickened by a diffuse exudate made up chiefly of polymorphonuclears. Cocci were scattered throughout the exudate (Fig 6) but only occasional ones were phagocytosed by polymorphonuclears.

Virulence

Bacteremia and meningitis occurred only in infection with the strain of gonococci which had been "modified" by passage on the chorio-allantoic membrane. This immediately raises the question of a possible change in "virulence." The early bacteriologists who worked with gonococci were wont to test the specificity of their cultures by inoculation in human beings. In this way four investigators found that the gonococci preserved their pathogenicity over a number of transfers in appropriate artificial media. Bumm (9) produced urethritis with the 20th generation, Anfuso (10) with the 7th, and Wertheim

(11) after 27 days of culture Finger *et al* (12) had studied one strain which maintained its virulence after more than 4 months of artificial cultivation

Early in our work on drug therapy of membranial infection (4) it was found that strains of organisms inoculated in similar doses varied in their ability to live and multiply on the membrane. This is not dependent solely on previous duration of growth on artificial media, for one strain when placed on the membrane immediately after cultivation from the urethral discharge failed to kill the embryo, and persisted in smears and cultures for only 3 to 4 days. The type strain of *N gonorrhoeae*, isolated in 1937, furnished by the American Type Culture Association, could be recovered from the membrane after 2 or 3 days incubation.

That virulence or killing power may vary with adjustment to continued growth on the membrane is shown by Table II. The percentage of 10 day embryos killed within 3 days after membranial inoculation is taken as a measure of the virulence. The strain of gonococcus used in this experiment, on isola

TABLE II

Membrane transfer	No of embryos inoculated	No dead in 3 days	Percentage dead
0	15	4	27
14-16	12	5	42
17-26	28	23	82
31-35	8	8	100

tion from the human cases failed to kill 10 day embryos. It had been grown on blood plates for about a month before serial chick passage was undertaken. It was then transferred from membrane to membrane at 2 day intervals with only an occasional blood plate transfer.

Since it is known that the gonococcus can be gradually adapted to adverse conditions, this may well represent an adaptation to the embryo, and death may have been caused by increased capacity for rapid multiplication rather than any change in toxicity or invasiveness.

With this change it was noted that the colonies obtained by culture of the 17th membranial passage had become very sticky so that they were hard to suspend in saline. Smears showed an abnormal amount of mucoid material. Capsular material could not be demonstrated by Hiss's method. This change in the gonococcus is also demonstrable in the older embryos inoculated intra amniotically as seen in Table III.

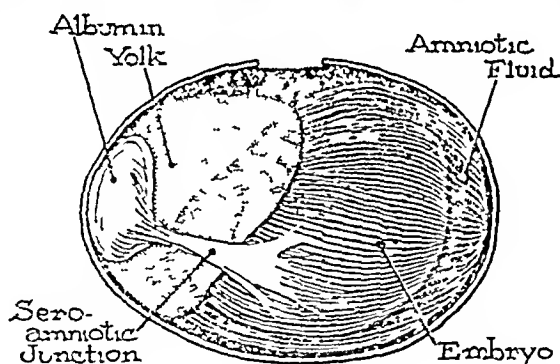
Strain L B killed a greater number of embryos in less time after it had become adjusted to the embryo. This difference is probably not significant in itself but agrees with the other data.

Embryos inoculated intra amniotically frequently recovered from the in

fection by the 2nd day, and later hatched. This may be partially due to the development of conditions unfavorable for the gonococci. The sudden release of albumin into the amnion which takes place following the rupture of the sero-amniotic junction (Text-fig 1) on the 14th day (13) may make the amniotic fluid less favorable for the growth of gonococci. This is in keeping with the failure of gonococci to multiply in the albumin of infertile eggs, while they ap-

TABLE III

Strain	Time of culture on artificial media	Age of embryo	No. inoculated	No. dead	Mortality
					<i>per cent</i>
Type L B	4 yrs	14	12	4 (3 days)	33
	1-2 wks	14	20	5 (2 days)	25
	After 15 membranal transfers	14	30	13 (18 hrs)	43
A W	2 mos	15	13	3 (2 days)	23
E M	2 days	13	13	5 (1 day)	38



TEXT-FIG 1 Diagram of 14 day chick showing amniotic cavity, and sero amniotic junction. Made from embryo hardened *in situ* by boiling.

parently are able to multiply in the yolk (14). Attempts to grow gonococci in unheated amniotic fluid of 10, 12, and 15 day embryos were unsuccessful.

Comparison with Meningococci

Susceptibility of the chick embryo to experimental infection by both meningococci (8, 15) and gonococci brings out certain interesting differences between these two closely related organisms.

1 The meningococcus kills susceptible embryos quickly but the gonococcus is often not fatal even to 10 day embryos

2 Meningococci usually invade the membrane of 10-day old chicks, localize on the endothelium of blood vessels and spread from there to the embryo itself. Gonococci rarely penetrate the 10 day membrane and thus rarely spread to the embryo

3 Meningococci introduced intra amniotically into 14 to 15 day embryos produce septicemia and often meningitis. Gonococci only occasionally gain the blood stream in small numbers, and meningitis is rare

These differences in biological behavior thus correspond to the differences in disease-producing qualities of the two organisms in the human host

Pathology of Acute Urethritis

Little has been added to our knowledge of the pathology of acute gonorrheal urethritis since the classical work of Finger, Ghon, and Schlagenhauser in 1894 (12). They inoculated three convicts intra urethrally with pure cultures of gonococci 2 and 3 days before execution and then obtained sections of the urethra. At first the gonococci occurred in scattered groups over the epithelium, particularly in the lacunae. They penetrated the connective tissue only where the epithelial layer was absent. Leucocytes pushed through the epithelial layer and engulfed an occasional gonococcus. By 3 days there was a massive inflammation and a great number of gonococci, most of which were phagocytosed. Many others were found in rows between the columnar epithelial cells of the pars pendula and also around the desquamated squamous cells of the thickened epithelial layer. They did not penetrate deeper into the tissues. Otherwise the process was entirely similar to any acute infection.

Study of a case (V 28 76) of gonorrheal urethritis at the Vanderbilt Hospital of 3 weeks duration revealed essentially the same findings. Smears had been found positive 2 days before the patient's accidental death. Bacteria were easily demonstrable scattered between the epithelial cells and in the leucocytic exudate (Wright's and Gram's stains). However they were also found clumped together in columnar epithelial cells. A slight vacuole surrounded the bacteria and separated them from the cytoplasm of the cell. We have been unable to demonstrate gonococci within the epithelial cells of the chick embryo.

The infection of the chorio allantoic membrane of the 10 day chick reproduces all of the essential characteristics of acute urethritis in man. Following the initial multiplication and spread of bacteria on the surface of the membrane there are a leucocytic exudate, a desquamation of epithelium (Fig 9) and later phagocytosis which may be partial or complete. A thickening of

the squamous ectoderm, and a deep inflammation in the mesoderm in the presence of few or no bacteria are characteristic

• Infection of the amniotic cavity allows the bacteria to spread to the embryo, occasionally to produce bacteremia and meningitis

SUMMARY

1 The reaction of the chick embryo to nine strains of gonococci was studied. Four of these were inoculated intra-amniotically in 14 to 15 day embryos.

2 Infection of the 10 day chorio-allantoic membrane was localized and accompanied by polymorphonuclear leucocytic phagocytosis.

3 Infection was either transitory or persisted until hatching. Several strains killed the 10 day embryo in 3 days.

4 Chicken immune sera placed on the infected membranes failed to affect phagocytosis.

5 Slight sinusitis, bronchitis, and infection of the pulmonary alveoli were common following intra-amniotic injection of 14 to 15 day embryos. Infection also extended to the pulmonary and abdominal air sacs.

6 Serial passage on the membrane so modified one strain that it killed a greater percentage of both 10 day and 14 day embryos. The infection after modification was also accompanied by bacteremia and meningitis in one embryo.

7 The ability of meningococci to invade tissue and produce septicemia is contrasted with the inability of gonococci to produce more than a superficial infection.

8 The infection of the embryo with gonococci reproduces all of the essential characteristics of the disease in man.

9 Gonococci were found within columnar epithelial cells in a case of acute human urethritis.

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EXPLANATION OF PLATES

PLATE 16

FIG 1 24 hour infection of chorio-allantoic membrane Note slight ulceration
Hematoxylin and eosin $\times 225$

FIG 2 Section from border of 5-day old infection of chorio allantoic membrane
Note plastic exudate Hematoxylin and eosin $\times 120$

FIG 3 48 hour infection of chorio allantoic membrane Ulcer followed inoculation with bacterial loop Note hyperplasia of ectoderm Hematoxylin and eosin $\times 36$

FIG 4 Lung of 14 day chick embryo inoculated intra-amniotically Infection is 72 hours old Note exudate in air sac at *A*, in bronchus at *B*, and in alveoli at *C*
Wright's stain $\times 36$

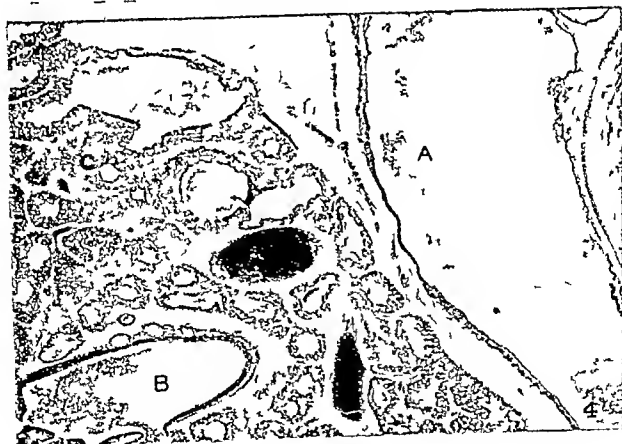
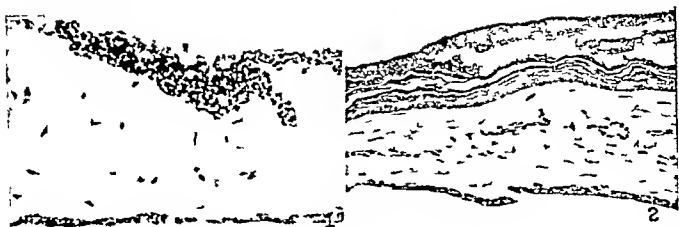


PLATE 17

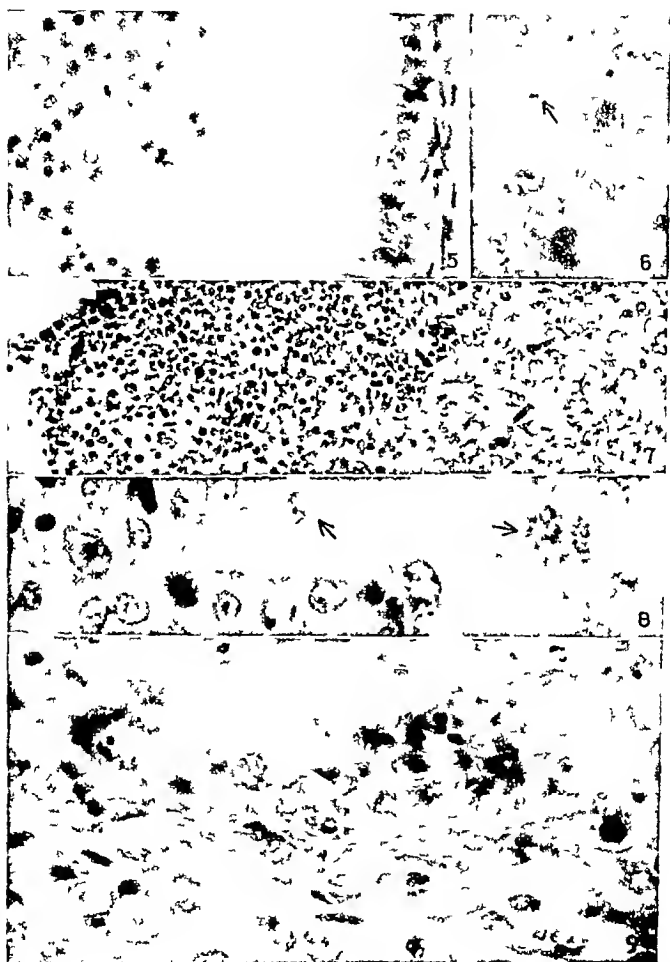
FIG 5 Enlargement of Fig 4 Exudate in air sac Wright's stain $\times 600$

FIG 6 Enlargement of Fig 7 Arrow points to diplococci Wright's stain $\times 2000$

FIG 7 Meningitis in 14 day embryo infected intra amniotically Infection 72 hours old Wright's stain $\times 250$

FIG 8 48 hour infection of 14-day old chick inoculated intra amniotically Arrows point to clumps of bacteria in alveoli Wright's stain $\times 2000$

FIG 9 Detail of Fig 3 48 hour infection of chorio allantoic membrane Note ulceration of thickened ectoderm Polymorphonuclears are seen penetrating the ectoderm Hematoxylin and eosin $\times 1600$



(Bang: *Gonococcus* infection of chick embryo)

TRANSPLANTABLE EPITHELIOMAS OF THE LIP AND MOUTH OF CATFISH*

I PATHOLOGY TRANSPLANTATION TO ANTERIOR CHAMBER OF EYE AND INTO CORNEA

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PLATES 18 TO 22

(Received for publication, July 2, 1941)

Among the neoplasms of the lower animals special interest is attached to those which have their counterpart in man and which lend themselves to experimental study. One such tumor is an epithelioma of the lip and mouth in the catfish (*Ameiurus nebulosus*). Like cancer of the lip in man it is of common occurrence,—at least in the region about Philadelphia—and hence easily available. Despite their high incidence we have been unable to find any record of these tumors in the literature. There has been observed, however, an epithelioma of the mouth in another species of catfish (1), and epitheliomas of the lip have been reported in several widely different species of both fresh water and salt water fishes (2-7). Since the literature on neoplasms in fish will be fully reviewed elsewhere no further details need to be given here.

The present paper deals in the first part, with the appearance and the behavior of the naturally occurring tumors, and with the relation of blood vessels to their development. The exposed position of the tumors and the ease with which catfish adapt themselves to laboratory conditions make possible prolonged study of the naturally occurring neoplasms, particularly with respect to their inception and course. An advantage, in this connection is the fact that in the catfish neither the normal nor the neoplastic oral mucosa undergoes keratinization, hence these epitheliomas are relatively translucent. There is thus afforded an opportunity to investigate the relation of the blood vessels to the inception and the progress of the cancer.

The second part is concerned with the results of transplantation of the tumor into the anterior chamber of the same and of other species, and with transplantation between the layers of the cornea.

Experimentally, the epitheliomas are readily transplantable into the anterior

* This study has been aided by a grant from the International Cancer Research Foundation.

chamber and—by taking advantage of an anatomic peculiarity (8)—between the two layers of the cornea. In these locations the growth of the tumor transplants may be followed by direct microscopic examination. These experiments extend to a new material our studies on neoplastic growth (9).

Material and Methods

During the past two years 166 live, tumor-bearing catfish were received in this laboratory. Most of them came from the Delaware and Schuylkill rivers in or near Philadelphia.¹ The fish were usually taken in nets and owing to injuries acquired during capture and transportation the mortality was high. Nearly one-half of the fish died within a few weeks after their delivery to the laboratory. A considerable number, however, were maintained in good condition for periods up to 9 months. They were kept individually or in groups of 3 or 4 in large aquaria, some in standing water which was replaced infrequently, others in tanks in which there was a continuous flow of fresh water. On the whole, aquaria with standing water proved more suitable. Food, consisting of earthworms and shredded liver, was offered at weekly intervals, but was often refused. The colony was inspected daily and fish showing signs of sickness were promptly removed and sacrificed.

The course of the tumors was followed by periodic examination supplemented by photographs. For this purpose, as well as for operations, the fish were wrapped in wet towels (leaving the eyes and the mouth exposed) and fastened to an examining board. Removal from the water was tolerated for periods of hours without apparent injury to the fish.

Much information was gained from examination of the living tumor, through the slit lamp microscope or modifications of this instrument. It thus became possible to observe directly the vascular pattern and the earliest stages of the new growth. The translucency of the oral mucosa and of the tumors was enhanced by the application of a few drops of glycerin. The blood vessels were brought into sharp relief by passing the beam of light through a green filter.

For transplanting tumor into the anterior chamber of the same and of alien species the technique employed was the same described in earlier papers (9). It should be noted however that the cornea of catfish has certain important peculiarities. Whereas in most vertebrates the transparent structures which compose the cornea are firmly fused and not readily separable, in catfish and certain other fish the cornea is readily separable into two layers (8). Of these, the inner layer is continuous with the sclera, and the outer with the skin. Between these two distinct and rather dense layers lies loose distensible areolar tissue. In making an operative incision at the sclerocorneal

¹ Many of the tumor-bearing fish and several hundred normal fish which were used for experimental purposes have been furnished us through the kindness of Mr C. R. Buller, Chief Fish Culturist of the State Fish Hatchery, at Bellefont, Pennsylvania, John J. Wopart, Jr., Superintendent of the State Fish Hatchery at Torredale, Pennsylvania, and Dr. Robert O. VanDeusen, Director of the Philadelphia Aquarium. We wish to express our grateful appreciation to these gentlemen for the aid and advice given.

junction, the blade of the knife readily passes through the outer corneal layer and may then easily be deflected into the subjacent loose connective tissue forming a small pocket within the cornea.

The structural peculiarity of the catfish cornea thus allows us to introduce transplants between the layers of the cornea instead of into the anterior chamber as has been done in previous experiments. This constitutes a new method for transplanting tumors and has the advantage that the intracorneal tumor fragments are at once fastened in place by the tissue so that they cannot float as often happens in the anterior chamber, thus natural conditions are more closely approximated.

As in previous studies the fate of the transplanted tumors was observed by direct microscopic examination (9). The observations of the living tumors whether naturally occurring or transplanted, were supplemented by the study of histologic sections prepared from tissues fixed in Susa or formalin solutions embedded either in celloidin or paraffin and stained with hematoxylin eosin or with Masson's trichrome mixture.

In the animals which were sacrificed, the internal organs were carefully inspected for evidence of disease and the existence of metastases.

Description of the Naturally Occurring Tumors

The tumors usually occur as large fleshy, protruding masses on the lips or the adjacent dental plates,² less often they involve other parts of the mouth or the skin. Thus in 166 tumor bearing animals the lips and dental plates were the sites of the new growths in 160. In this group, additional tumors were noted, in the oral cavity (at some distance from the labial tumors) in 10 animals on the barbels in 7 the head in 29 and elsewhere on the body in 5 animals. In a smaller group of 6 animals lips or dental plates were not affected, and the tumors, usually of large size, occurred on the head.

All of the neoplasms are similar in structure and take their origin from the labial, or oral mucosa, or from the skin which in catfish has the character of a mucosal surface. The tumors in the different locations may be regarded as expressions of the same neoplastic disease.

Appearance and Distribution of Tumors of the Lip and Dental Plates—The appearance of representative tumors is shown in Figs. 1 to 5. Their surface is usually smooth (Fig. 1) or coarsely nodular (Fig. 3). Their shape varies, some are round or oval, others form flattened tubular masses. All arise from a broad base which merges into the surrounding tissue. The tumors contrast sharply with the much paler mucosa by their reddish color. Their consistency is firm, resilient or even rubbery. There is no tendency to undergo ulceration, although superficial abrasions are commonly seen. Most of the tumors meas-

The dental plates of catfish lie in immediate contact with the lips. They are flat ridges slightly elevated above the remainder of the oral mucosa which covers them. They support multiple rows of tiny primitive teeth which barely project through the mucosal surface.

ure from 1 to 1.5 cm in their greatest diameters, but some tumors are so massive (3 cm or more) as to prevent closure of the mouth. The cut surfaces are moderately bloody, and in most tumors soon become covered with a sticky oozing mucus. The stroma is inconspicuous and usually cannot be recognized clearly with the unaided eye. There is no sharply defined boundary at the base of the tumors, although extensive downward invasion is uncommon.

In our series the catfish tumor was solitary in 57 of the animals, two tumors were present in 39, and more than two tumors in 64.

The distribution of the tumors is summarized in Table I. It is seen that the lower lip or dental plate is somewhat more frequently affected than the upper lip or dental plate, and that in approximately one-half of the cases, both lips or dental plates are involved. The tumors have no favored site on these structures, and affect the different regions with approximately equal frequency.

Of the animals having tumors on both lips or dental plates 53 (i.e., 60 per cent) have the tumor in direct apposition. Thus in Fig. 2 is shown a massive

TABLE I
Distribution of Tumors on Lips or Dental Plates

Regional distribution of tumors			Apposition tumors
	No of animals	Per cent	
Upper lip or dental plate only	30	18.1	Of 89 animals having tumors of both lips or dental plates 53 (i.e., 60 per cent) have the tumors in apposition.
Lower lip or dental plate only	41	24.7	
Both lips or dental plates	89	53.6	
Neither lip involved	6	3.6	

primary tumor on the lower lip in apposition to a much smaller secondary growth, in Fig. 3 the lower lip is seen to be the site of two tumors, one on each lateral half, and opposite each, smaller tumors have developed. We shall revert to such appositional tumors, the development of which we have been able to observe repeatedly.

Histopathological Structure—The tumors consist of closely packed masses of epithelial cells supported by a delicate but richly vascular stroma (Fig. 6). The majority of the cells are columnar or polyhedral and have abundant cytoplasm. The nucleus is vesicular and contains one or two prominent nucleoli (Fig. 7). Mitoses are uncommon, but cells with two or more nuclei are frequently seen. No cytoplasmic or nuclear inclusions are present. The growth is obviously an epithelioma.

The cells are irregularly arranged. In the more central parts of the crowded cell nests, structures resembling epithelial "pearls" are occasionally seen. However, keratinization does not take place. In contrast to the normal mucosa, clavate cells are absent from most of the tumors and sense organs are never seen. Clusters of mucus-containing cells are found in many tumors, they are usually packed in the central areas of the cell masses.

At first most of the tumors grow in an outward direction and show little sign of invasion. Later, broad pegs of tumor cells commonly push deeply into the subjacent tissues, frequently extending to the bony structures. These masses of tumor cells in most areas, have a well preserved basement membrane. However, as the tumors become larger they become more and more invasive, and extend as flame shaped processes into the stroma (Fig 8). The cells now become larger, their staining qualities alter and they lose their compact arrangement (Fig 9). When this stage has been reached, strands of cells are not infrequently found to have pushed into vessels, where they form emboli (Figs 10 to 12).

The stroma of the tumor is scanty, but is well supplied with vessels which usually are widely and irregularly dilated and have thin, capillary like walls. In many tumors there is a scattering of small round cells, probably lymphocytes, such collections are most prominent in the basal parts of the growths.

Appearance and Distribution of Tumors in Oral Mucosa and on Skin—These tumors have the same general character as those on the lip. An exception, is the almost black appearance of some cutaneous tumors (Fig 5), the color of which is due to an abundance of pigment cells in the supporting stroma.

Nearly all of the growths occur at no great distance from the lips, i. e. on the oral mucosa at the base of the barbels or on different parts of the head. Most of the growths are discrete, but some are obviously extensions from the primary tumors of the lips or dental plates. The tumors in the oral cavity are the most interesting. In all but one animal they were located on the floor of the mouth (Fig 4), and at some distance from the lips. Since the lip tumors are often deeply invasive, penetrating into vessels and forming emboli, the question arises whether the growths of the oral mucosa or skin are metastatic in origin. It is quite possible that those in the mouth cavity are, but we do not believe, at present, that any of the cutaneous tumors have arisen by metastatic dissemination of tumor cells. Rather, we regard them as independent of the tumors of the lips. This view is based upon the occurrence of large cutaneous tumors in 5 animals which had no other tumors. The close similarity of the catfish skin to the mucous membrane of the mouths makes it seem likely that the same tumor producing agent can lead to the development in both skin and mucous membrane, of similar types of new growths. Secondary tumors in the internal organs have not yet been observed.

Incidence, Geographical Distribution, and Seasonal Variation—Fishermen of experience who have furnished us most of the tumor bearing fish have estimated their incidence at approximately one in 300 to 500 catfish taken from the Delaware or Schuylkill rivers, in or near Philadelphia. A closer estimation of the incidence is difficult because tumor bearing catfish appear to be abundant in certain streams and pools and absent in others. No information is as yet available as to the geographic distribution. Seasonal variations in incidence and in character of tumor have not been observed.

Size, Condition, and Sex—All of the tumor-bearing catfish were well grown and measured between 25 and 35 cm. No tumors were found in small, that is to say young, fish. Most of the tumor-bearing fish were in good condition and showed no other signs of disease. An exception were fish with massive growths which prevented closure of the mouth; these fish usually were emaciated. Of 50 fish in which sex was determined by examination of the gonads 23 were males and 27 females, hence the factor of sex appears to play no part in this neoplastic disease.

Course of the Tumors Relation of Blood Vessels to the Development of Tumors

In Table II are summarized the records of observations on the course of tumors in 15 catfish which were studied for periods of from 6 to 36 weeks. It is seen that the rate of growth of the tumors was relatively slow. A representative example is the first tumor on the list which during 20 weeks increased only from 15×6 mm to 18×8 mm, in its two greatest horizontal diameters. Indeed, in several of the tumors no significant change in size was noted during periods of approximately 3 months. There were, however, tumors which grew rapidly. Two examples are shown in Text-fig. 1. In one of these, the tumors more than doubled in size within a month (upper figure), in the other they became massive within 10 weeks.

The figures also illustrate the development of appositional tumors. In 5 of the 15 catfish of this series, and in a much larger number which died in less than 6 weeks, we were able to watch the beginning and the further development of new tumors, all of them in apposition to established tumors.

The earliest visible evidence of neoplastic change was the establishment of a more or less circumscribed patch of intense hyperemia on the mucosa. At this time the mucosal surface was entirely smooth and showed no sign of proliferation. Within approximately 2 weeks the hyperemic mucosa began to thicken, and became slightly elevated. Within 1 or 2 months, the local thickening had progressed to the formation of a definite nodular or flattened growth.

Direct microscopic examination permitted more detailed study of the vascular phenomena and brought out the fact that profound alterations of the blood vessels accompany the development of the epithelioma. At present, our earliest microscopic observations have extended only to the stage in which epithelial proliferation has begun, i.e., after the tissue has been hyperemic for a week or so. The vessels were markedly dilated and formed irregular, wide meshed anastomosing nets in contrast to the uniform, small capillary loops of the adjacent normal mucosa (Figs. 13 and 14). The caliber of the vessels in the neoplastic zone varied greatly, some channels were dilated, bulbous, and had a sinusoidal character while their continuations were contracted, many of the proliferating vessels had coarse walls, others appeared unduly delicate. In spite of the abnormality of the vessels, blood was seen to flow through them swiftly, as was indicated by the rapid passage of the erythrocytes. The atypical appearance of the vascular pattern persisted as the tumors became fully developed (Figs. 15 and 16).

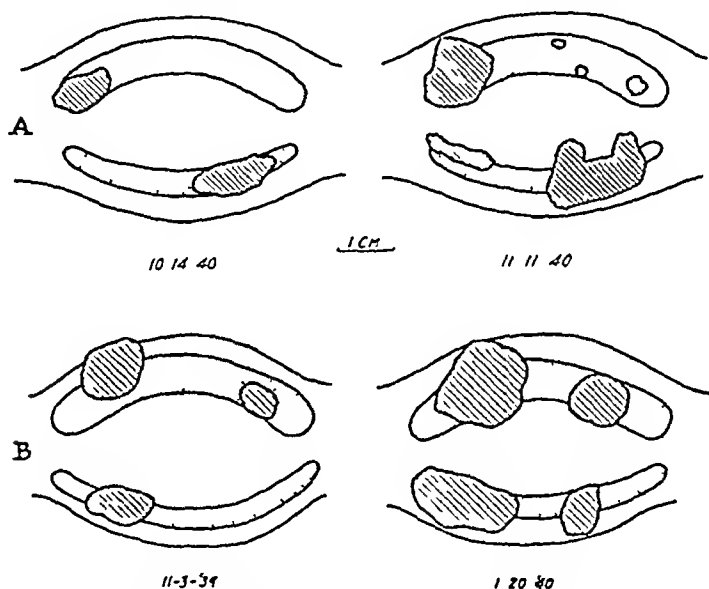
TABLE II
Course of the Tumors

This table summarizes records of observations on 15 tumor bearing fish which have been kept for at least 6 weeks. Sizes at the beginning and at the end of the periods of observation are given in millimeters and refer to the two greatest horizontal diameters. Vertical diameters are omitted because of the difficulty of measuring accurately the depth of the tumors.

Designation of tumor	Duration of observation	Course
	<i>weeks</i>	
1	20	Solitary tumor on lower lip 15×6 slowly increased to mass measuring 18×8 mm
14	22	Apposition tumors on upper lip 10×10 on lower lip 5×5 . The larger tumor increased to 15×10 the smaller remained stationary. 4 new tumors each 2×3 appeared on the upper dental plate
37	10	Two tumors on upper lip and dental plate one 15×10 the other 7×5 . In apposition to larger tumor is a growth 9×5 . The tumors on upper lip grew to 20×15 and 15×10 respectively the apposition tumor to 20×15 . A new tumor developed in apposition to the smaller mass on upper dental plate and attained a size of 10×10
108	36	Tumor on upper lip and dental plate 20×12 mm in apposition a tumor 12×5 . Laterally to each of the original lesions new apposition tumors developed during the 5th month of observation these increased slowly attaining a size of 5×5
110	19	Solitary tumor on lower dental plate and lip. No definite changes in size no new tumors developed
116	12	Two tumors on lower lip and adjacent dental plate each 12×10 which increased only slightly in size. In apposition to one of these was a small tumor which grew from 2×2 to 8×4 mm
118	17	Two apposition tumors in corner of lip 12×10 and 6×4 respectively. In addition two tumors on floor of mouth 15×10 and 8×4 respectively. No definite change in size
123	6	Solitary tumor on upper lip 12×10 . Within 3 wks after the beginning of observations a new tumor developed in apposition attaining a size of 3×2
129	9	Solitary tumor on dental plate 11×8 which remained stationary. In apposition to it a new tumor developed which slowly increased to 3×2
130	9	Two tumors each 9×9 on upper lip and dental plate in apposition to one of these was a tumor 11×10 on lower dental plate. No significant change
133	13	Very large confluent tumors, 25×10 which involved almost the entire upper lip and dental plate these increased slightly. In apposition was a mass 17×10 which did not undergo significant changes
134	13	Very large tumor 27×10 on upper dental plate which progressed slowly. In apposition were 2 smaller tumors, one of which grew from 2×2 to 7×3 mm the other remained stationary
137	12	Large tumor 17×10 on upper lip and dental plate which was in apposition to a smaller growth 9×8 . Neither tumor changed significantly
141	10	Solitary tumor on lower lip and dental plate, 10×8 . No definite change in size
144	11	Tumor on lower lip and dental plate 13×6 in apposition was a growth 11×9 . Both remained unchanged

Our studies on the relation of blood vessels to the development of the epithelioma are incomplete, and are being continued. Here, all we wish to emphasize is that neoplastic growth was preceded by a vascular reaction and that the blood vessels in the tumor were atypical. The possible significance of these phenomena will be briefly discussed.

Interpretation of Vascular Phenomena—The relation of blood vessels to tumor growth has been investigated by a number of students of cancer, usually by means of injection preparations (10 - 13). A remarkably complete study



TEXT-FIG 1 Course of established tumors and development of new tumors in apposition with the primary growths. The upper pair of drawings is from an animal not included in Table II, the lower pair is from catfish 37 of the table. In each drawing the single curved lines represent the outer contour of the lips, and the stippled band the adjacent dental plates.

of this kind is that of Thiersch (10), who through injections of numerous human skin cancers recognized that the vascularity of the early tumor is greatly increased over that of normal skin. Later Boll (11) took up this problem and "from a study of early epitheliomas and of the growing edges of such tumors concluded that changes in the blood vessels determined the proliferation of the epithelium." Goldmann (12) in experimental tumors also found profound vascular reactions at the very beginning of epithelial proliferations. In these and similar investigations living tumors have not been used. In a recent paper Ide, Balcer, and Warren (14) studied the vascularization of transplants of rabbit epithelioma as seen in the transparent rabbit ear chamber.

None of these studies has elucidated the nature of the relation between

increased blood supply and neoplastic growth (15) The catfish epithelioma appears to be favorable material for such an investigation in the living animal

Transplantation of the Epithelioma of the Lip

The experiments on transplantation fall into 5 groups in which a total of 148 animals were used In the first group, small bits of the epitheliomas were implanted into the anterior chamber of the eye of the tumor bearing animals themselves In the second group, the tumor was put into the anterior chamber of two different species of fish, and in the third, into the anterior chamber of leopard frogs Groups 4 and 5 deal with autotransplants and homotransplants between the layers of the cornea

The arrangement and general results of the experiments are summarized in Table III They will now be taken up in order

TABLE III

Summary of Transplantation Experiments of Catfish Epithelioma

Species of recipient animal	No of tumors transplanted	No of animals	Results
Catfish (autotransplants into anterior chamber)	14	14	Progressive growth of transplants in 10 of the animals
Goldfish sunfish (heterotransplants into anterior chamber)	6	38	No growth Transplants remained translucent for several weeks then regressed
Leopard frogs (heterotransplants into anterior chamber)	6	47	No growth Prompt exudation around transplants which soon became opaque and rapidly regressed
Catfish (autotransplants into cornea)	7	7	Progressive growth of transplants in 6 of the animals
Catfish (homotransplants into cornea)	5	42	Progressive growth of transplants in 4 of the experiments

Autotransplantation into Anterior Chamber of the Eye—In a series of 14 animals, 10 showed progressive growth of the transplant Usually attachment occurred within a week and by the end of the 2nd week vascularization had begun The pattern of growth was relatively simple During the 2nd or 3rd week, proliferating cells spread from the tumor fragment to the under surface of the cornea forming broad membranes (Fig 17) which gradually thickened and acquired a delicate vascular stroma If vascularization occurred early the main mass of the transplant remained viable and gradually increased in size Growth continued in this manner until by the end of the 2nd month the anterior chamber was filled with a compact, well vascularized mass of tumor tissue (Fig 18) Thereafter the growth became stationary and then slowly regressed

Occasionally, the growing tumor invaded the iris (Fig 19) In 3 cases it

extended into the loose scar tissue of the operative wound at the sclerocorneal junction, forming a mass which protruded beyond the cornea. In one case the transplant passed into the posterior chamber where it grew well. In several instances strands of tumor tissue grew over the anterior surface of the lens where they could be seen as delicate white fibers. Vascularization in this region is late and these outgrowths soon regressed.

Heterotransplantation into the Anterior Chamber of the Eye—Repeated attempts to transplant the catfish epithelioma into the anterior chamber of alien species of fish, or to frogs were unsuccessful (Table III). Interesting differences, however, were noted in the local reaction of the recipients to the foreign tumor, and in the fate of the latter. In goldfish or sunfish, implantation of the catfish tumor led to no exudative reaction, and the aqueous humor remained clear. The fragments of transplanted tumor remained translucent and apparently survived for as long as 4 weeks, they then gradually became opaque, and within 8 weeks regressed completely.

On the contrary when transplanted to members of another class, the leopard frogs, an abundant leucocytic exudate promptly formed around the transplant. The exudate was sharply localized and the aqueous humor remained clear. Regression was usually very prompt and the transplants were resorbed entirely within 3 weeks.

Transplantation between the Two Layers of the Cornea—The results of autotransplantation and homotransplantation were similar in this series (Table III). In both groups transplants from one of the tumors rapidly regressed. In the remainder, progressive growth occurred, in 6 of the 7 autotransplants, and in 64 per cent of five tumors inoculated into non-tumor-bearing catfish.

There was no significant exudation of cells following the implantation of the tumor fragments. The cornea in all animals remained clear and translucent. The transplants were at once firmly fixed in place through the mechanical factor of tissue tension. We were now able to observe the influence on the pattern of growth when the tumor is placed in a tissue rather than in space filled with fluid, as is the case in the anterior chamber.

A notable difference was the more prompt fibrous attachment and vascularization of the corneal transplants. More striking was the difference in the mode of growth. There were no broad surfaces of contact within the cornea over which the outgrowth could spread in one plane. Rather the transplants grew expansively by proliferation of cells throughout the mass. Room for the growing tumor was achieved by further splitting of the corneal layers which became widely separated, one from the other. The growing margin of the tumor was usually quite smooth, here and there, however, projections extended outward, creeping into the clefts between the layers of the cornea, but this was never extensive (Figs 20 and 21). None of the tumors eroded through either of the corneal layers (Fig 22).

The rate of growth was approximately the same as in the anterior chamber. Within less than 2 months after implantation a compact mass of tumor greatly distended the cornea (Figs 23 to 28) pushing its outer layer forward. With maximal distention of the cornea, growth became slow or ceased entirely, but no evidence of regression was noted during the 3 months period of observations.

SUMMARY

The catfish (*Ameiurus nebulosus*) taken from streams near Philadelphia, is commonly afflicted with an epithelial tumor bearing some resemblance to epithelioma of the lip in man. This neoplasm usually occurs as solitary or multiple, large, red, fleshy masses upon the lips or dental plates and by reason of its size, may prevent closure of the mouth. The tumor is comprised of epithelial cells, often in papillary arrangement, supported by a delicate vascularized connective tissue stroma. The larger growths frequently invade adjacent normal tissues and force their way into vessels where they are found as emboli. The clinical course of the tumor is one of relatively slow but progressive growth.

This neoplasm has been observed from the time of its inception in a number of animals. Thus it has been learned that the proliferative stage of the neoplastic process is preceded and accompanied by a striking vascular reaction. Intense hyperemia invariably occurs in that region of the mucosal surface which later becomes the site of neoplastic proliferation. Furthermore, by direct microscopic observation of the living tumors the atypical structure and arrangement of the blood vessels become apparent. A study of the significance of these vascular phenomena in their relation to the inception and growth of the tumor is now in progress.

It has been found possible to transmit the catfish tumor to fish of the same species by implanting fragments of the tumor into the anterior chamber of the eye. Also, by taking advantage of an anatomical peculiarity of the catfish cornea, it has been possible to embed the tumor fragments in normal tissue where it could still be readily observed both in the gross and microscopically. The growth of the transplants in the eye has been followed by periodic examination of the living tumor by means of the slit lamp microscope. In the anterior chamber the tumor characteristically forms dense membranes which spread over the inner surface of the cornea. In this manner growth continues until the tumor fills the chamber. Between the two layers of the cornea, tumor growth is expansive.

Attempts to transplant the tumor to the anterior chamber of two other species of fish and to frogs were unsuccessful. Implantation of the catfish epithelioma in alien species of fish excited no exudative response, whereas in a less closely related species of animal the leopard frog a pronounced exudative reaction resulted.

The frequent natural occurrence, the exposed anatomical position, and the facility of experimental transmission to the eye combine to make the catfish tumor especially suitable for studies upon neoplasia

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EXPLANATION OF PLATES

Figs 1 to 5, 13 to 16, and 18 to 22 are unretouched photographs of living tumors, the remainder are sections which were stained with hematoxylin and eosin All magnifications are approximate Most of the figures were photographed by Mrs Miriam R Barrett

PLATE 18

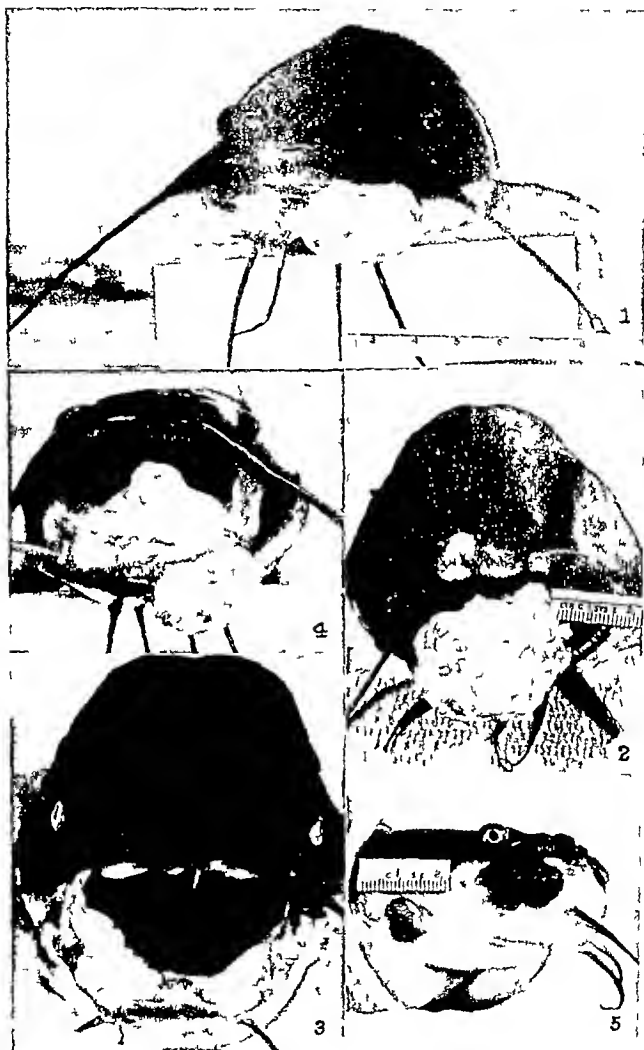
FIG 1 Epithelioma of the lip The tumor projects as a prominent, fleshy mass having a smooth surface beneath which numerous, large vessels may be seen

FIG 2 A large, lobulated tumor involves the lower lip and has extended back wards into the oral cavity In direct apposition to this primary growth, a smaller tumor has developed on the upper lip and the adjacent dental plate The masses prevent the closure of the mouth

FIG 3 On the lower lip are located two flattened, lobulated tumors In direct apposition two small tumors have developed

FIG 4 Mouth cavity of tumor bearing catfish shown in Fig 1 Upon opening the mouth two additional tumors were found, one of which had arisen from the dental plate (shown as a granular surface adjoining the tumor), the other was located on the floor of the mouth at some distance from the lip

FIG 5 In addition to an epithelioma on the upper lip, there are two discrete tumors on the side of the head They have the same general structure, and differ only in their darker color which is due to the presence of pigment-bearing cells in the supporting stroma



(Lucké and Schlumberger Transplantable epitheliomas of catfish I)

PLATE 19

FIG 6 Sections through a representative tumor showing compact masses of epithelial cells which are supported by a scanty vascular stroma (the blood vessels are collapsed and hence not distinct in the photograph) $\times 200$

FIG 7 The epithelial cells of the tumor at higher magnification are seen to have no orderly arrangement $\times 480$

FIG 8 Boundary zone of tumor and subjacent tissue Flame shaped processes of the tumor dip downward into the loose connective tissue of the lip $\times 260$

FIG 9 Epithelioma invading the subjacent tissue The masses of epithelial cells have lost their compact texture and numerous, small processes are invading the deeper connective tissue $\times 200$



(Lucke and Schlumberger Transplantable epitheliomas of catfish I)

PLATE 20

FIG 10 A tumor embolus lying in a vein at some distance from a primary tumor on the lower lip which had extended into the deeper tissues, and destroyed a portion of the mandible $\times 260$

FIG 11 Another tumor embolus in a vein from the case shown in the preceding figure $\times 520$

FIG 12 A tumor embolus lying in a venule (The embolus came from the invading tumor shown in Fig 8) $\times 260$

FIG 13 Vasculature at the junction of normal mucosa (upper half of photograph) and developing tumor (lower half of photograph) as seen in the living animal The transition between normal and abnormal vascularization is well shown In the normal mucosa the vessels appear as uniform small capillary loops In contrast, the vessels at the margin of neoplastic development are tortuous, of unequal caliber, and bear bulbous dilatations $\times 70$

FIG 14 Vascular change similar to that shown in the preceding figure from another incipient new growth The intense hyperemia and the characteristic irregularity of the vascular pattern are well shown $\times 70$

FIG 15 Vasculature of a fully developed tumor showing a prominent, irregular network of vessels $\times 70$

FIG 16 A field from another large tumor in which are seen the great irregularity of caliber and distribution of the vessels $\times 70$



PLATE 21

FIG 17 A broad membranous outgrowth from the transplanted tumor has covered the inner surface of the cornea 73 days after transplantation $\times 70$

FIG 18 Autotransplant in anterior chamber The entire chamber is filled by a dense, well vascularized mass 35 days after transplantation $\times 10$

FIG 19 Autotransplant in anterior chamber The photograph is taken in a slightly oblique position to show a membranous growth spreading from the edge of the proliferating tumor over the inner surface of the cornea, invading the iris, at the upper and lower poles 33 days after transplantation $\times 10$

FIG 20 Profile view of an intracorneal autotransplant The cornea is greatly distended with the tumor mass which has pushed the outer layer of the cornea forward From the periphery of the main mass strands of neoplastic cells may be seen infiltrating the cornea 60 days after transplantation $\times 10$

FIG 21 Front view of the tumor shown in the preceding photograph Vessels are seen to pass from the corium of the adjacent skin into the tumor $\times 10$

FIG 22 Profile view of an autotransplant between the two layers of the cornea, which have been widely separated by the compactly growing mass $\times 10$

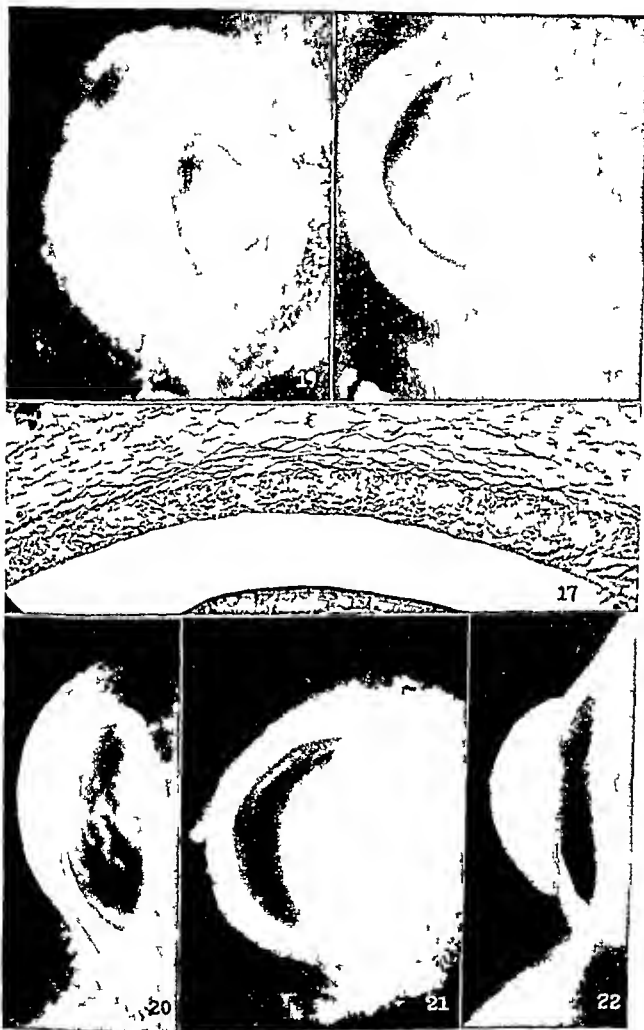


PLATE 22

A series of photographs of histologic sections to illustrate stages of growth of transplants between the layers of the cornea

FIG 23 Transplant immediately after operation to show its position in the cornea $\times 80$

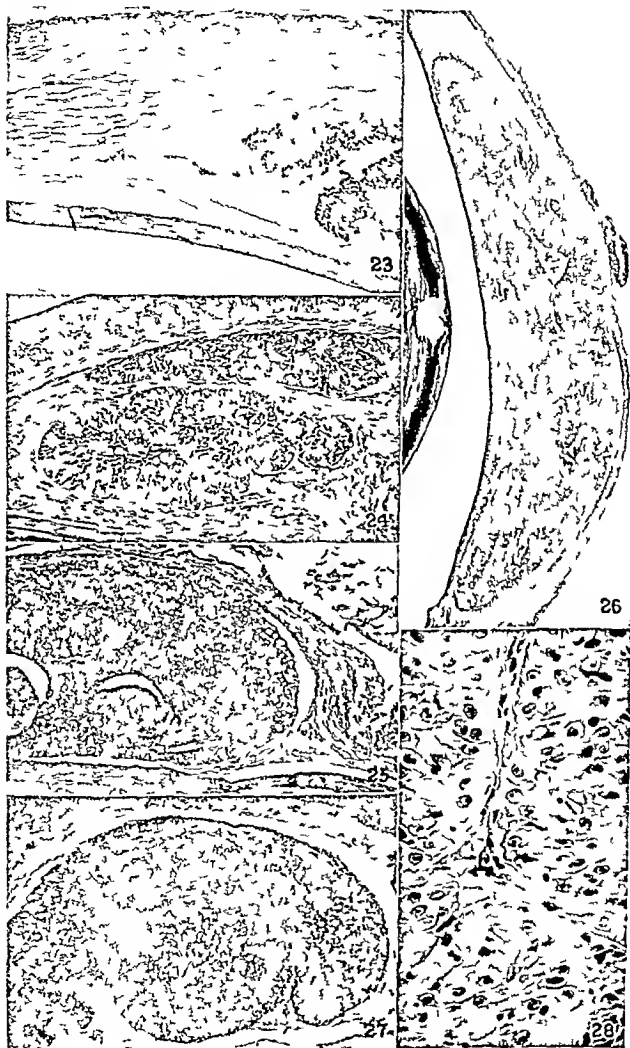
FIG 24 Early stages of growth 14 days after operation the transplant is firmly fixed in a stroma derived from corneal fibrous tissue, and vascularization has begun $\times 80$

FIG 25 69 days after transplantation the tumor has become compact and widely separates the layers of the cornea $\times 50$

FIG 26 A section of the transplant shown in Fig 22 The photograph shows the position of the tumor in the cornea and its relation to the anterior chamber and lens $\times 25$

FIG 27 A tumor 89 days after transplantation Clusters of mucus forming cells are seen in the central part of the tumor mass $\times 50$

FIG 28 A portion of the tumor shown in the preceding figure The arrangement is similar to that of the naturally occurring tumors Densely packed masses of neoplastic cells are supported by a delicate vascular stroma $\times 390$



(Lucké and Schlumberger Transplantable epitheliomas of catfish I)

THE COMPLEMENT FIXATION TEST IN THE DIAGNOSIS OF VIRUS INFECTIONS OF THE CENTRAL NERVOUS SYSTEM

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The application of the complement fixation reaction to the study of central nervous system virus infections has been complicated by the fact that infected brain or cord tissue is usually the only source of antigen. Brain tissue extracts have the property of being anticomplementary and can be used only when they are diluted and some of their components removed. Such procedures have resulted in a decrease of the final antigenic titre.

Howitt (1), although stating that the margin between specificity and non-specificity is narrow, reported specific complement fixation reactions with the viruses of equine encephalomyelitis, lymphocytic choriomeningitis, and St. Louis encephalitis. Several other workers have reported contradictory results with rabies virus. Greval (2) and Havens and Mayfield (3), using sheep and guinea pig hyperimmune sera respectively, obtain protocols sufficiently clear cut to permit evaluation of the specificity of the test. Because of the practical importance of a satisfactory complement fixation test for the diagnosis of central nervous system virus infections, renewed attempts have been made in this laboratory to resolve the complicating factors. The result of these attempts to the present time will now be described in detail,¹ together with a simple technique for carrying out complement fixation tests with brain virus antigens. Finally, the application of this test to the diagnosis of human virus encephalitides is reported.

Materials and Methods

Viruses—Rabies, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, Eastern equine encephalomyelitis, Western equine encephalomyelitis, louping ill, spontaneous encephalomyelitis of mice (Theiler's disease) and a mouse strain of human poliomyelitis (Armstrong) have been studied. These viruses are propagated in this laboratory by intracerebral mouse passage; rabies virus is propagated also by intracerebral passage in dogs, rabbits and guinea pigs.

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¹ Preliminary statements have been published in *Science* (4) and reported also at the Forty first Annual Meeting of the American Association of Pathologists and Bacteriologists held in New York in April, 1941 (5).

Antigens—Brain and sometimes the brain and cord are used as antigen. They have been prepared in several ways in an effort to eliminate anticomplementary effects. The different methods will be compared later, meanwhile, the procedure which we finally adopted as routine will be described. Fresh, infected brain tissue is weighed, ground up in a mortar, and emulsified in ten times its weight of a diluent consisting of 0.85 per cent saline containing 2 per cent normal, inactivated guinea pig serum. This emulsion is kept in the ice box for 20 hours and then centrifuged in a horizontal centrifuge at 2500 R P M for $\frac{1}{2}$ hour. The supernatant is then frozen and thawed five times in a dry ice-alcohol mixture, whereupon a flocculate appears. Finally it is centrifuged in a small angle head centrifuge for 1 hour at approximately 3500 R P M. After the addition of merthiolate in a dilution of 1:10,000, the supernatant is stored in the ice box ready for use. Antigens prepared in this manner are usually employed undiluted. Their titre varies according to the virus and the species of animal from which the virus derives, from 1:8 to 1:128. They have not proved anticomplementary when kept for periods of 2 months, on further aging, however, they have tended to become anticomplementary. By means of this method good antigens have been obtained from all viruses except mouse passage poliomyelitis (Armstrong strain) and occasional strains of rabies street virus. In these latter instances the virulence of the virus from which the antigen has been prepared has been low,—0.03 cc of the 10^{-3} dilution intracerebrally, at best, whereas all other viruses, including those of most strains of street rabies, have titred higher,—rabies street, 10^{-4} , spontaneous encephalomyelitis of mice (Theiler), strain GVII, 10^{-5} , St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, and louping ill, 10^{-6} or 10^{-7} , rabies fixed, 10^{-7} , and Eastern and Western equine encephalomyelitis, 10^{-8} .

Immune Sera—Hyperimmune sera have been obtained by injecting dogs, mice, rabbits, and guinea pigs with homologous infected brain tissue. In general the following procedure has been followed in the case of mice. A first injection of 0.5 cc of a 10^{-4} brain emulsion is given subcutaneously, after 1 week, intraperitoneal injections of 0.5 cc are given at 4 to 6 day intervals, starting with a dilution of 10^{-4} , following this with a dilution of 10^{-3} and then 10^{-2} , and repeating the last injection from three to five times. The number of injections has varied from five to ten and the length of the period of immunization from 20 to 30 days. The titre of virulence of the viruses used for immunization is determined at frequent intervals, as noted above.

This course of vaccination has proved fatal to a number of mice, especially when animals too young have been employed, however, by employing mice 3 to 4 months old at the time immunization is started, the proportion of deaths can be reduced to 10 per cent or less. In order to maintain a constant supply of immune sera, the mice were kept under immunization by repeated intraperitoneal injections of infected brain emulsions while they lived, sometimes as long as 6 months (repeated bleedings from the heart proved fatal in the end in most cases). These subsequent injections were administered at intervals longer than those of the initial immunization period, usually every 10 or 15 days. The sera contained complement fixing antibodies whenever tested, although the highest titre seemed to be reached at the 4th or 5th week, thereafter it diminished gradually.

Rabies hyperimmune sera in guinea pigs were obtained by means of five to ten intraperitoneal injections of infected brain tissue in dilutions from 10^{-4} to 10^{-2} , 1 cc being administered each time in rabbits from eight to fifteen injections of the same dilutions in 2 cc volumes were employed. All injections were given about 1 week apart.

Rabies hyperimmune sera in dogs have been difficult to obtain: three dogs were kept under immunization for a period of 3 months by means of weekly 5 cc injections of infected brain tissue in a dilution of 10^{-2} before they showed any complement fixing antibodies in their blood, and then only in low titre.

Sera from mice were obtained by bleeding from the heart under ether anesthesia and pooling the blood samples: the sera from other animals were drawn either from the heart or from one of the veins and always tested individually. For reasons to be discussed later, temperatures of inactivation for the different sera have been established as follows: for guinea pig sera, 56°C, for mouse sera, 60°C, and for rabbit and dog sera, 65°C. All sera were heated for 20 minutes. When inactivated at 65°C the sera were usually diluted 1:2 or 1:3 in order to prevent coagulation—which may occur with undiluted serum at this temperature.

Data on the smallest number of injections necessary to induce complement fixing antibodies, the promptness of their appearance, and their persistence in serum are not included herein; we have limited ourselves in this paper to a report of observations on the presence and specificity of the complement fixation reaction.

Complement—Fresh guinea pig serum constituted the complement. It was titrated in the presence of the antigens as indicated later, and 2 units were used in the test.

Hemolytic System—Sheep red blood cells and rabbit anti-sheep hemolysin constituted the hemolytic system. A 3 per cent suspension of packed cells in a volume of 0.25 cc plus 3 MND of hemolysin also in a volume of 0.25 cc was used. The cells and hemolysin were mixed $\frac{1}{2}$ hour before use in the test.

Reaction—The reaction was carried out in the following manner: 0.25 cc of antigen, 0.25 cc. of serum in serial twofold dilutions, and 0.5 cc of complement diluted to contain 2 units were incubated in the ice box for 18 hours and then left at room temperature for $\frac{1}{2}$ hour. The hemolytic system consisting of 0.5 cc. of sensitized sheep cells was then added. The total volume in each tube was thus 1.5 cc. The tubes were next incubated in a water bath at 37°C for $\frac{1}{2}$ hour and the reaction was read. Complete hemolysis was expressed as 0, absence of hemolysis as 4 plus with $\pm 1, 2$, and 3 plus indicating intermediate degrees of hemolysis. The titre of the serum was taken as the last dilution giving a 2 plus or better fixation.

Development of a Specific Complement Fixation Test with Brain Antigens

The technique outlined above was developed after considerable experimentation to eliminate anticomplementary effects of the antigens, non specific reactions between antigens and sera, and anticomplementary effects of sera. The various steps will now be described in detail.

Anticomplementary Power of the Antigens—Determination of the anticomplementary power of an antigen, necessarily the first and most important

step in all complement fixation tests, is even more essential in the case of brain extract antigens since their pronounced anticomplementary effect is well known, especially following long incubation of the first phase at low temperature. For this reason particular attention has been paid to this point.

In general the different methods to eliminate anticomplementary power have consisted either in the use of high dilutions of brain tissue or in extraction of the brain tissue with fat solvents, since the lipoids in the brain seem to be mainly responsible for the anticomplementary action. Of course these methods must be such that in reducing the anticomplementary action to a minimum the antigenicity remains unchanged.

In our hands, 10 per cent, 5 per cent, and 1 per cent brain emulsions in saline or distilled water merely centrifuged in the horizontal centrifuge have exhibited anticomplementary effect, if the brain tissue was further diluted this inhibitory effect was eliminated but then the antigenicity of the preparation was either very low or non-existent. Consequently the method of bringing the brain tissue to high dilutions proved unsatisfactory.

We have tried the technique described by Howitt (1), in which the brain tissue is dried from the frozen state and this procedure followed by ether extraction. Although the resultant antigens were not anticomplementary, their antigenicity was low in all cases and usually no better than 1:2 to 1:4.

The supernates of brain emulsions, when spun in the ultracentrifuge at speeds varying from 10,000 to 30,000 R.P.M. for 1 hour and especially if filtered through a Seitz pad, showed no anticomplementary power and were used to advantage. Filtration through a Seitz pad dispensed successfully with all non-specific effects, although it did reduce the antigenicity to a variable degree.

Filtration of a brain emulsion through a Seitz filter, either directly without centrifugation or following centrifugation in the horizontal centrifuge, proved to be very unsatisfactory, the rate of filtration was extremely slow and the filtrate of very low antigenicity or entirely devoid of it.

From a comparison of these different methods of preparing brain tissue antigens we have determined that the technique described under materials and methods gives the highest antigenic titre without anticomplementary effects and at the same times dispenses with constant use of the ultracentrifuge. The freezing and thawing of the brain emulsion are done not so much to disrupt the cells and extract more antigen as to produce marked flocculation, thus making subsequent centrifugation in the angle head centrifuge more effective.

As shown by Bedson and Bland (6) long incubation of the first phase at low temperature is preferable to incubation at 37°C for 1/2 or 1 hour to detect complement-fixing antibodies associated with some virus infections. This has also been our experience and on this account prolonged incubation in the ice box has been adopted. However, this treatment has the disadvantage of increasing the anticomplementary power of the antigens. Hence it has been found neces-

sary to determine the titre of the complement in the presence of the antigens at the beginning and at the end of the period of incubation of the first phase in order to estimate more precisely the titres obtained

Titration of the complement and determination of the anticomplementary power of the antigens were carried out in the following manner

A preliminary titration of the complement was done by placing in test tubes increasing amounts, from 0.05 cc to 0.5 cc of fresh guinea pig serum in a dilution of 1:30 and completing the volume to 1 cc in each tube with saline. Then the hemolytic system was added and the whole incubated at 37°C for 1½ hours. This gave a preliminary titre of the complement. Following this preliminary titration the anticomplementary power of the antigens was determined in duplicate.

Two sets of tubes, each set having as many series of six tubes as there were antigens plus an extra series for saline alone, were set up. Increasing amounts of diluted complement from 0.05 cc to 0.5 cc were placed in each series of tubes, completing the volume to 0.75 cc in each tube with saline. Then 0.25 cc of a given antigen was added to each tube of the series of six in both sets, a series in each set being reserved for saline instead of antigen. The first set of tubes was incubated at 37°C for 1½ hours, the second incubated in the ice box for 18 hours along with those of the test proper. After incubation at 37°C the first set of tubes received the hemolytic system and it was again incubated at 37°C for another ¾ hour period. This latter titration gave the titre of the complement in the presence of the antigens and determined the amount of complement to be used in the final test. Moreover, it revealed any gross anticomplementary action that might have taken place in the antigens. The second set received the hemolytic system at the end of the 18 hour incubation period.

The real value of the procedure described above was determined by the incubation of the tubes of second titration at low temperature in conjunction with the test proper, for it disclosed not only anticomplementary properties in some antigens that appeared suitable on first titration but at the same time indicated the actual amount of free complement present in the tubes when the hemolytic system was added. This second titration replaced the routine antigen control tube and provided more accurate information. After repeated trials we now know that antigens prepared as described above do not show any anticomplementary effect at the end of an 18 hour incubation period. The titre of the complement is usually the same and sometimes even better than that in the tubes with saline and no antigen.

Table I presents in detail the result of one experiment in which the anticomplementary power of several antigens was determined. After incubation at 37°C for 1½ hours, the titre of the complement in the series with saline alone was represented by 0.10 cc. Of all antigens tested only No. 4 was anticomplementary in the gross and had to be discarded, this was a 10 per cent crude brain emulsion centrifuged at low speed with no further treatment. The remaining antigens, namely, 1 per cent and 5 per cent brain emulsions

centrifuged at low speed, an antigen prepared according to the method described by Howitt, and three antigens prepared according to our method, were not anticomplementary. After incubation in the ice box for 18 hours, however, the titre of the complement in saline was 0.13 cc. Preparations 2 and 3 which at 37°C were not anticomplementary proved to be strongly anticomplementary after standing in the ice box 18 hours, as in the case of antigen 4 which had al-

TABLE I
Titration of Complement in Presence of Several Antigens

Material employed as antigen		Incubation of first phase											
		½ hr at 37°C						18 hrs in ice box (2°C)					
		Amount of complement in dilution of 1:30											
		0.20 cc	0.16 cc	0.13 cc	0.10 cc	0.08 cc	0.065 cc	0.20 cc	0.16 cc	0.13 cc	0.10 cc	0.08 cc	0.065 cc
No 1	No antigen saline	0	0	0	0	1	3	0	0	0	±	1	2
No 2	Mouse brain 5 per cent Centri- fuged 500 R P M for 5 min	0	0	0	0	1	2	3	4	4	4	4	4
No 3	Mouse brain 1 per cent Centri- fuged 500 R P M for 5 min	0	0	0	0	1	2	1	2	3	4	4	4
No 4	Dog brain 10 per cent Centri- fuged 2000 R P M for 10 min	0	0	3	4	4	4	4	4	4	4	4	4
No 5	Dog brain 10 per cent Centri- fuged 15,000 R P M for 45 min	0	0	0	0	±	1	0	0	0	±	1	3
No 6	Mouse brain 10 per cent—frozen and thawed Swedish centrifuge for 1 hr	0	0	0	0	±	1	0	0	0	0	±	1
No 7	Dog brain 10 per cent—frozen and thawed Swedish centrifuge for 1 hr	0	0	0	0	±	1	0	0	0	±	1	3
No 8	Dog brain 1 per cent—Howitt's method	0	0	0	0	±	1	0	0	0	±	1	2

0 indicates complete hemolysis

± " no hemolysis

±, 1, 2, and 3 indicate intermediate degrees of hemolysis

ready proved anticomplementary at 37°C. Antigens prepared in accordance with our method, together with that of Howitt, did not become anticomplementary.

The use of antigens such as Nos. 2 and 4 in a complement fixation test would not be suitable because of their anticomplementary action, that of No. 3 would be inadvisable even though the antigen control tube showed hemolysis because its specific action would be too close to the non-specific zone to be of any great significance. Titration in the ice box gave a better estimate of the reaction than the antigen control tube. In the final specific tests we have used 2 units of complement as indicated by the titration at 37°C. When there are several

antigens in one test, as may often be the case, there may be differences in the titre of the complement as given in the presence of each antigen. These differences are generally not referable to more than one tube in the series and in such cases the 2 units of complement are computed from the antigen giving the lowest titre—an amount equivalent to $2\frac{1}{2}$ or $2\frac{3}{4}$ units with the remaining antigens.

Non-Specific Reactions of Normal or Immune Sera—Our immune sera were obtained with few exceptions by injection of infected homologous brain tissue. As shown by Witelsky and Steinfeld (7), injection of heterologous brain tissue gives rise, at least in certain species, to organ specific antibodies which rule out the use of such sera for complement fixation tests in which brain extracts are used as antigens. But even when homologous brain tissue was used for the production of hyperimmune sera, a major difficulty arose in our work because of the property in sera from some species of reacting in a non-specific manner with brain tissue extracts, a reaction similar to that described by Takenomata (8) for bacterial cultures and filtrates, and by Mackie and Finkelstein (9) for a number of substances such as cholesterol, peptone, diluted alcohol, etc.

Takenomata showed that the serum of normal rabbits has the property of fixing complement in the presence of bacterial cultures and filtrates—that the reaction was more pronounced when the first phase was incubated at 37°C rather than at 0°C and that this property of the sera could be destroyed by heating at 62°C . Mackie and Finkelstein described a similar property for sera from normal individuals of several species. Such sera could fix complement in the presence of cholesterol, peptone, alcohol, and other substances, whereas sera from other species either did not possess this property to equal extent or lacked it entirely. This power of fixing complement was in general destroyed by heating the sera at 60°C and in most instances was greatly reduced by heating at 56°C .

As noted above, antigens which were not anticomplementary could be obtained from brain tissue. However, as a result of tests for complement fixing antibodies on sera from different animal species, it soon became apparent that so much non specificity was present that the reaction was rendered valueless. Sera from mice, rabbits, dogs, and human beings exhibited to a variable extent, the property of reacting with brain extracts and gave a complex which bound complement.

Table II shows the extent of this non specific reaction. Normal sera and immune sera from several different species were tested against antigens prepared according to the standard method. The column under homologous antigens shows the result of the reaction between the immune sera and the antigens containing the same virus as that used for immunization of the animals. The column under heterologous antigens gives the result with one of the unrelated antigens. Three rabbit sera, one normal, another rabies immune, and the third Eastern equine encephalomyelitis immune, reacted with

both antigens. Although the immune sera showed a higher titre with the corresponding antigen, the degree of non-specificity was too high to be of any diagnostic use. On the other hand, neither the sera alone nor the antigens were anticomplementary. Mouse rabies immune, Eastern equine encephalomyelitis immune, and Japanese B immune sera also exhibited a degree of non-specific reaction but not as high as that shown by rabbit sera. Two human convalescent sera, Eastern equine encephalomyelitis and St. Louis encephalitis,

TABLE II

Non Specific Complement Fixation Shown by Sera from Different Animal Species When Tested with Mouse Brain Extracts as Antigens
Sera inactivated at 56°C for 20 minutes

Sera	Homologous antigens								Heterologous antigens								No antigen saline			
	Sera in dilutions																			
	1 3	1 6	1 12	1 24	1 48	1 96	1 192	1 3	1 6	1 12	1 24	1 48	1 96	1 192	1 3	1 6	1 12	1 24		
Rabbit normal	—	—	—	—	—	—	—	4	4	2	±	0	0	0	0	0	0	0		
Eastern equine encephalomyelitis immune	4	4	4	4	4	3	0	4	4	4	3	±	0	0	0	0	0	0		
Rabbit rabies immune	4	4	4	4	4	3	0	4	4	4	3	1	±	0	0	0	0	0		
Mouse Eastern equine encephalomyelitis immune	4	4	4	2	0	0	0	2	±	0	0	0	0	0	0	0	0	0		
Mouse rabies immune	4	4	4	4	1	0	0	3	1	0	0	0	0	0	0	0	0	0		
Japanese B immune	4	4	4	3	1	0	0	4	3	0	0	0	0	0	0	0	0	0		
Human St Louis convalescent	4	4	4	±	0	0	0	4	4	±	0	0	0	0	0	0	0	0		
Human Eastern equine encephalomyelitis convalescent	4	4	4	4	4	2	0	4	4	4	2	0	0	0	0	0	0	0		
Guinea pig rabies immune	4	4	4	4	4	2	0	0	0	0	0	0	0	0	0	0	0	0		
No serum control of antigens	0	—	—	—	—	—	—	0	—	—	—	—	—	—	0	—	—	—		

— indicates not tested

Other footnotes as in Table I

reacted with both antigens but not when saline was substituted for the antigens. Finally, guinea pig serum was the only one that gave an entirely specific reaction.

It was found possible to eliminate this non-specific reaction by employing as antigen a brain emulsion filtered through a Seitz pad, following centrifugation at high speed. But because of the reduction in titre of the antigen thus obtained, and especially to eliminate constant use of the ultracentrifuge, a different method was applied, namely, subjecting the sera to be tested to the action of heat (4, 5).

Sera from various animal species as well as from human convalescents were heated for periods of 20 minutes at temperatures of 56°C (the usual temperature of inactivation which destroys complement present in the sera) 60°C, and 65°C. In some instances higher temperatures—70°C and 75°C—were also applied. When temperatures of 65°C or higher were employed, the sera were ordinarily diluted to avoid

TABLE III

Effect of Heating Sera at Different Temperatures on Specific and Non-Specific Complement Fixing Antibodies

Sera	Non-specific reaction with heterologous antigen. Sera heated for 20 min at temperature					Specific reaction with homologous antigen. Sera heated for 20 min at temperature				
	56 C	60 C	65 C	70 C.	75 C	56 C	60 C.	65 C.	70 C.	75 C.
Rabbit 1 normal	1 16*	1 8	0							
2 rabies immune	1 12	1 6	0	0	0	1 96	1 96	1 48	1 24	0
3	1 32	1 32	0			1 64	1 64	1 32		
4 Eastern equine encephalomyelitis immune	1 24	1 24	0			1 96	1 96	1 48		
Rabbit 5 Western equine encephalomyelitis immune			0					1 4		
Mouse lymphocytic choriomeningitis immune	1 3	0				1 48	1 48			
Mouse rabies immune	1 6	0	0			1 24	1 12	0		
Japanese B encephalitis immune	1 6	0	0			1 24	1 24	0		
Human No 1 louping ill convalescent	0	0				1 16	1 16			
Human No 2 Eastern equine encephalomyelitis convalescent	1 48	1 24	0			1 96	1 96	1 48		
Human No 3 St Louis encephalitis convalescent	1 16	1 8	0			1 32	1 16	1 8		
Human No 4 Wassermann positive	1 48	1 12	0							
Guinea pig rabies immune	0	0	0			1 24	1 12	1 6		
Dog rabies immune	1 32	1 8	0			1 64	1 32	1 16		

* Highest dilution of serum giving a 2 plus or better reaction first dilution 1 1 1 2 or 1 3

coagulation. Table III presents a summary of the results of several tests. This table shows the highest dilution of serum giving a 2 plus or better reaction with the homologous and a heterologous antigen. The first dilution of the sera was 1 1, 1 2, or 1 3. Rabbit sera gave a non specific reaction in dilution as high as 1 32; this reaction remained practically unchanged at 60°C but disappeared at 65°C. On the contrary, the reaction with the homologous antigen persisted with only slight decrease at 65°C. At 70°C there were still antibodies left although of lower titre. It was only following heating at 75°C that the sera were no longer active. With mouse sera the non

specific reaction present at 56°C disappeared at 60°C, whereas the specific reaction persisted practically unchanged at 60°C and even at 62°C in some cases. Antibodies in the mouse sera were lost at 65°C. Some of the human sera did not give a non-specific reaction at 56°C and others did. This non-specific reaction was especially noted in Wassermann-positive sera, although others, such as Nos. 3 and 4, which were not Wassermann-positive, likewise showed a non-specific reaction at 56°C and 60°C, but not at 65°C. The specific reaction shown by the human sera was not altered by heating at 60°C and 65°C. Whether there was a great loss of titre after heating at 65°C is difficult to say because of the fact that the reaction was masked in part by the non-specific effect, the drop in titre shown by some sera, for example No. 3, may be more apparent than real. A dog rabies immune serum reacted similarly to rabbit sera. And finally, with guinea pig immune sera there was never a non-specific reaction following inactivation at 56°C, the specific antibodies were still present at 60°C, with accompanying loss of titre, they were usually no longer present at 65°C and never at 68°C.

These observations indicate that there is a margin sufficiently wide between the temperature capable of destroying elements in the serum responsible for the non-specific reaction and that materially affecting specific antibodies. By observing this varying resistance to heat it has been possible to obtain a specific reaction. As indicated above, temperatures of inactivation have been established at 56°C for guinea pig sera, 60°C for mouse sera, and 65°C for rabbit and dog sera, all sera being heated for 20 minutes.

For human sera a temperature of 56°C has seemed to be sufficient in most cases, but in order to eliminate slight degrees of non-specificity a temperature of inactivation of 60°C has been adopted except in cases of Wassermann-positive sera or those sera that for some unknown reason give a non-specific reaction at this temperature. In such cases the temperature of inactivation has been 65°C. If enough serum is available, the best procedure is to carry out the reaction with the serum in duplicate, inactivating one sample of serum at 60°C and the other at 65°C.

Anticomplementary Power of the Sera—Some rabbit, dog, and human sera, especially when kept in the ice box for some time, may have an anticomplementary effect of their own, that is, they may fix complement in the absence of antigen. This power may be present when the serum is diluted as much as 1:4 or 1:6 but heating at 60°C or 65°C destroys the anticomplementary effect of the serum.

Results of Newly Developed Complement Fixation Test with Hyperimmune Sera

Hyperimmune Sera from Mice—Mouse hyperimmune sera prepared as described above were tested against homologous and heterologous antigens. The result of a single test is summarized in Table IV.

Each serum was tested against each one of the antigens. The test was done in duplicate, one set with sera being inactivated at 56°C, the other set with sera at

60°C Only the reaction at 60 C has been reproduced here Its specificity is complete There was no cross reaction in this test between the St Louis No 3 virus and the Japanese B strains the serological relationship between these two viruses has been investigated by Webster (10) by means of the neutralization test with the result that they have been shown to be serologically different even though cross neutralization not exceeding 1 to 10 M.L.D. may be present Whether there is any slight degree of cross reaction in the complement fixation test is being further investigated, although if present, it would appear to be very insignificant The serum of lymphocytic

TABLE IV

Complement Fixation Test with Mouse Immune Sera Inactivated at 60°C for 20 Minutes Mouse Brain Antigens

Antigens	Sera								
	St Louis encephalitis No 3	Japanese B encephalitis No 2604	Japanese B encephalitis No 17	Japanese B encephalitis No 12	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Louping ill	Rabies (street)	Rabies (fixed)
St Louis encephalitis No 3	1 32*	0	0	0	0	0	0	0	0
Japanese B encephalitis No 2604	0	1 32	1 32	1 64	0	0	0	0	0
Japanese B encephalitis No 17	0	1 32	1 64	1 128	0	0	0	0	0
Japanese B encephalitis No 12	0	1 32	1 128	1 128	0	0	0	0	0
Lymphocytic choriomeningitis	0	0	0	0	1 128	0	0	0	0
Eastern equine encephalomyelitis	0	0	0	0	0	1 64	0	0	0
Louping ill	0	0	0	0	0	0	1 64	0	0
Rabies (street)	0	0	0	0	0	0	0	1 32	1 8
Rabies (fixed)	0	0	0	0	0	0	0	1 16	1 8
No antigen	0	0	0	0	0	0	0	0	—

* Highest dilution of serum giving a 2 plus or better reaction first dilution 1 4

choriomeningitis immune mice has a high titre of complement fixing antibodies which is in contrast to the uniform absence of neutralizing antibodies in the blood of these animals Louping ill immune serum has a high titre of antibodies Finally rabies fixed and street viruses react with each other

In another test presented in Table V two additional viruses were studied for their capacity to elicit complement fixing reactions the Western equine encephalomyelitis and the mouse spontaneous encephalomyelitis viruses The sera were used in this test undiluted and in increasing twofold dilutions the temperature of inactivation being 60 C for 20 minutes With the viruses of Eastern equine encephalomyelitis and Western equine encephalomyelitis there was no cross reaction The GVII strain of mouse spontaneous encephalomyelitis gave a high titre serum

Hyperimmune Sera from Guinea Pigs—Guinea pigs have been used to obtain rabies immune serum. Thus far seven animals have been immunized with the result that all except one have shown antibodies in high titre in dilutions varying from 1/48 to 1/382. Some of them required no more than three or four injections to give a positive serum. When tested against a normal brain antigen or against an unrelated antigen (and this is done in every individual test), guinea pig serum has always given a specific reaction following inactivation at 56°C.

Hyperimmune Sera from Rabbits—Rabies, Eastern and Western equine encephalomyelitis immune sera have been prepared in rabbits. The Eastern and Western equine encephalomyelitis sera were adapted originally for neutralizing antibodies by inoculation of mouse rather than rabbit infected brain

TABLE V

*Complement Fixation Test: Mouse Immune Sera Inactivated at 60°C for 20 Minutes
Mouse Brain Antigens*

Antigens	Sera		
	Eastern equine encephalomyelitis	Western equine encephalomyelitis	Spontaneous encephalomyelitis of mice
Eastern equine encephalomyelitis	1/4*	0	0
Western " "	0	1/16	0
Spontaneous encephalomyelitis of mice (Theiler's disease)	0	0	1/64

* Highest dilution of serum giving a 2 plus or better reaction: first dilution, 1/1

issue. Only two injections of a 1 to 1,000 suspension were given to each of two rabbits, 2 cc each time. The animals received no further treatment and after 1 year their sera were tested for complement-fixing antibodies, although it may be added that a specific reaction was not expected on account of the injection of heterologous (mouse) brain virus. These two sera, when tested in dilutions of 1/2 or 1/3, have given an occasionally non-specific reaction, even following inactivation at 65°C, probably as a result of the presence of some mouse protein antibody, in higher dilutions the reaction has been specific at all times. In Table VI are given the results of cross-reactions between the two sera and a rabies immune serum at three different temperatures of inactivation.

The Western equine encephalomyelitis serum was tested undiluted and in serial twofold dilutions against Western and Eastern equine encephalomyelitis and rabies antigens. The titre of this serum was very low, 1/4, although it was completely specific. The Eastern equine encephalomyelitis immune serum inactivated at 65°C had a titre of 1/48 with the homologous antigen, but in a dilution of 1/3 it reacted

also with rabies antigen. Finally, the rabies immune serum at 65 C reacted only with the rabies antigen.

The production of rabies immune sera in rabbits has not been an easy task. In most instances no antibodies were present in the first 4 or 5 weeks of immunization, by which time from five to seven injections had been given. A total of nine rabbits have been immunized with the Pasteur strain of rabies virus propagated in the same species.

Table VII gives the titre of all sera as obtained in a single test. The immunization of the individual rabbits had been started at different times, so that at the time of

TABLE VI

Rabbit Immune Sera. Complement Fixation Tests Following Inactivation for 20 Minutes at Different Temperatures

Antigens	Sera inactivated at temperature of						
	56 C		60 C		65 C		
	Eastern equine encephalomyelitis immune	Rabies immune	Eastern equine encephalomyelitis immune	Rabies immune	Eastern equine encephalomyelitis immune	Rabies immune	Western equine encephalomyelitis immune
Eastern equine encephalomyelitis	1/96*	1/24	1/96	1/12	1/48	0	0
Western equine encephalomyelitis	—	—	—	—	0	0	1/4
Rabies	1/24	1/96	1/24	1/96	1/3	1/48	0

Eastern and Western equine encephalomyelitis rabbit immune sera were obtained by injecting mouse infected brain.

* Highest dilution of serum giving a 2 plus or better reaction.

— = not tested.

bleeding for the test they had received differing numbers of injections and their immunization had been in progress for varying lengths of time. The number of injections received by each rabbit as well as the time elapsed from the beginning of immunization are shown in the table. Here it may be seen that all reactions at 56 C and 60 C were non-specific. Following inactivation of the sera at 65°C, six rabbits gave a specific reaction, another rabbit No. 4, gave a negative reaction, and rabbits 5 and 9 a non-specific one. The sera of these two latter rabbits were tested at even higher temperatures of inactivation and also with antigens filtered through a Seitz pad, with negative results. The reaction remained non-specific. These animals had been immunized with tissue that had remained in the ice box for a period of 5 to 7 days, while in most other cases the infected tissue used for immunization was fresh. This possible explanation is being studied further.

In general, with respect to hyperimmune sera from eleven rabbits the different resistance of the specific and non-specific antibodies to heat was consistent.

Hyperimmune Sera from Dogs—The complement fixation reaction with dog sera has been difficult because of the presence of non-specific antibodies similar to those encountered in rabbit sera and also because of the hemolysins for sheep cells that may occur spontaneously. Moreover, dogs do not seem to

TABLE VII

Rabbit, Rabies Immune Sera Result of Complement Fixation Test Following Inactivation of Sera for 20 Minutes at Different Temperatures

Sera			Temperature of inactivation of sera								
			56°C			60°C			65°C		
Rabbit No	No of injections	Period of immunization	Rabies antigen	Heterologous antigen	No antigen	Rabies antigen	Heterologous antigen	No antigen	Rabies antigen	Heterologous antigen	No antigen
		mos									
1	31	12	1 48*	1 24	0	1 48	1 24	0	1 24	0	0
2	8	2	1 24	1 24	0	—	—	—	1 12	0	0
3	15	6	1 12	1 12	0	1 6	0	0	1 3	0	0
4	11	5	1 24	1 24	0	1 6	1 6	0	0	0	0
5	11	5	1 96	1 96	0	1 96	1 96	0	1 24	1 48	0
6	9	4½	1 24	1 24	0	1 12	1 12	0	1 6	0	0
7	9	4½	1 48	1 24	0	1 48	1 24	0	1 24	0	0
8	9	4½	1 24	1 24	0	1 24	1 6	0	1 12	0	0
9	9	4½	1 96	1 96	0	1 96	1 96	0	1 48	1 24	0

* Highest dilution of serum giving a 2 plus or better reaction first dilution, 1 3

TABLE VIII

Dog, Rabies Immune Sera Result of Complement Fixation Test Following Inactivation of Sera at 65°C for 20 Minutes Mouse Brain Antigens

Antigens	Dog 2 26 serum in dilutions								Dog 2 31 serum in dilutions								Dog 2 32 serum in dilutions							
	1	2	4	1	8	1	16	1	32	1	64	1	2	4	1	8	1	16	1	32	1	64		
Rabies, mouse	—	4	1	0	0	0	0	4	4	4	4	3	0	—	4	4	1	0	0	0	0			
Japanese B encephalitis, mouse	—	0	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0			
No antigens	—	0	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0			

— = not tested

Other footnotes as in Table I

respond to the immunizing injections as readily as do guinea pigs or rabbits. Three dogs have been under immunization treatment for a period of nearly 6 months, their sera being tested at frequent intervals. The first definite specific reaction was obtained at 3 months after approximately twenty injections had been given.

Table VIII shows the result of a test with the sera of the three dogs. The sera were inactivated at 65°C for 20 minutes following dilution to 1 2. The reaction with

rabies antigen gave titres of 1 4 1 32, and 1 8 respectively whereas there was no reaction with the unrelated antigen (Japanese B encephalitis)

Results of Newly Developed Complement Fixation Test with Sera from Cases of Central Nervous System Virus Infections

Animal Sera—Mice intramuscularly infected with 0.01 cc. of rabies street virus did, in some instances, have complement fixing antibodies coincident with the development of paralysis. Also mice intracerebrally inoculated with the virus of spontaneous encephalomyelitis (Theiler), that developed paralysis but survived for 30 days, showed antibodies in their blood in titres from 1 2 to 1 8. Finally, preliminary studies with the virus of St. Louis encephalitis indicate that, following a single 0.5 cc. intraperitoneal injection of virus in a dilution of 1 100, about one third of the mice have antibodies as early as the 6th day and all mice have antibodies in fairly high titre by the 13th day.

The sera of dogs receiving injections of 0.5 cc. of street virus in a dilution of 1 20 into the neck muscles (11) have been tested, thus far with negative results.

Human Sera—Smadel, Baird, and Wall (12) showed that in a proportion of human cases of lymphocytic choriomeningitis complement-fixing antibodies were present, that they appeared early in the disease or the first stages of convalescence later they disappeared and were substituted by neutralizing antibodies. This seems to be the only instance in which complement fixing antibodies in the blood of human beings have been described in connection with any of the diseases herein considered. Accordingly we applied our test to human cases of central nervous system virus encephalitis which had previously been diagnosed on the basis of neutralization tests. Thus far, few such cases have been available and most of these old ones.²

Tests with human sera are summarized in Table IX. All cases of Eastern and Western equine encephalomyelitis and louping ill tested gave a positive specific reaction, the persistence of louping ill antibodies in the blood of an individual 8 years following infection is striking. Only recent cases of St. Louis encephalitis and lymphocytic choriomeningitis have shown complement fixing antibodies.

In addition, several other human sera have been tested, among them six sera from patients with St. Louis encephalitis from the 1933 and 1937 epidemics, three from patients with lymphocytic choriomeningitis one of them infected

The sera from human cases have been kindly supplied by the following persons: Dr. W. Lloyd Aycock, Dr. Margaret G. Smith, Dr. G. O. Brown, Dr. Charles D. May, Dr. Morgan B. Hodskins, Dr. Horace L. Hodes, Dr. Richard E. Shope, Dr. Sherman F. Gilpin, Dr. Roy F. Feemster, Dr. R. V. Platou, Dr. Albert B. Sabin, Dr. Walter O. Klingman, Dr. Charles F. Branch, Dr. Thomas Henley, Dr. Joseph Johnson, Dr. Herald R. Cox, and Dr. Joseph L. Lilienthal, Jr. To all we express our gratitude.

5 years previous to the present bleeding, and the other two recent ones, and finally, about fifteen sera from patients with indefinite central nervous system symptomatology, none of which has, to our knowledge, been diagnosed. These sera have been tested against Eastern and Western equine encephalomyelitis,

TABLE IX
Complement Fixation Tests with Human Sera

Sera				Antigens (mouse brain)						
No	Diagnosis of disease by neutralization test	Interval between disease and bleeding for complement fixation test	Temperature of inactivation for 20 min	Louping ill	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Western equine encephalomyelitis	St Louis encephalitis	Japanese B encephalitis	No antigen saline
1	Eastern equine encephalomyelitis	2 yrs	60		0	1 8*			0	0
2	" "	2 "	65	0	0	1 48		0		0
3	" "	2 "	65		0	1 8		0		0
4	" "	2 "	60		0	1 32		0		
5	" "	2 "	65		0	1 2		0		0
6	Western equine encephalomyelitis	3 "	65		0	0	1 8	0		
7	Western equine encephalomyelitis (?)	?	60		0	0	1 4	0		0
8	Western equine encephalomyelitis	2 yrs	60		0	0	1 1	0	0	
9	Louping ill	8 "	60	1 16	0	0			0	0
10	" "	4 "	60	1 4	0	0		0	0	0
11	Lymphocytic choriomeningitis	2 wks	60	0	1 4	0		0	0	0
12	" "	3 "	60	0	1 1	0		0	0	0
13	" "	2-4 wks	60		1 1		0	0		
14	St Louis encephalitis	4 mos	60			0	0	1 1		
15	" "	3 "	65		0	0		1 8		0
16	" "	3 "	60		0	0		1 1		0
17	" "	?	60				0	1 4		

* Highest dilution of serum giving a 2 plus or better reaction first dilution, 1 1 or 1 2

St Louis encephalitis, lymphocytic choriomeningitis, and some against louping ill antigens, all with negative results

COMMENT

The etiological diagnosis of the human encephalitides remains a difficult one. Thus far the clinician has had to depend on methods that are either unsatisfactory or else cannot be used as routine. Isolation of virus from the

cerebrospinal fluid or blood has been accomplished only in a few cases of lymphocytic choriomeningitis and, perhaps, Japanese B encephalitis. In general, though, virus is recoverable only from the central nervous system, thus providing only a postmortem diagnosis. In most of the cases in which a diagnosis has been possible, it has been brought about through the medium of the cumbersome neutralization test. The neutralization test has two disadvantages. In the first place, neutralizing antibodies do not appear in general until after convalescence is well under way so that the diagnosis is a delayed one. In the second place, routine use of the neutralization test is hardly possible in a diagnostic laboratory because of the complicated procedure involved.

The complement fixation test as described above appears eminently practical. The antigens are stable for a considerable period of time and the test procedure is, in general, a familiar one. Lastly, no animals are required and the result is apparent within a few hours.

SUMMARY AND CONCLUSIONS

A specific complement fixation test can be obtained in various central nervous system virus infections by using as antigens emulsions of infected brain tissue, freezing and thawing the brain emulsion, and then centrifuging it in an angle bead centrifuge at 3500 R.P.M. for 1 hour. The method has proved reliable in the case of rabies, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, Eastern equine encephalomyelitis, Western equine encephalomyelitis, louping ill, and spontaneous encephalomyelitis of mice (Theiler's disease).

The specificity of the reaction, regardless of the virus involved, requires different temperatures of inactivation of the sera according to animal species: 56°C for guinea pig, 60°C for mouse and 65°C for rabbit and dog sera, all heated for 20 minutes. For human sera a temperature of inactivation of 60°C also for 20 minutes has been adopted, at this temperature the reaction is in general specific.

Complement fixing antibodies in high titre were found in the sera of rabbits, guinea pigs, mice, and dogs immunized with rabies virus.

Complement fixing antibodies were present in high titre in sera drawn from two persons 8 years after an attack of louping ill, from five persons 2½ years after an attack of Eastern equine encephalomyelitis, and from two persons 2½ years after Western equine encephalomyelitis. In cases of St. Louis encephalitis and lymphocytic choriomeningitis, complement fixing antibodies have been found shortly following infection but not after long periods.

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TABLE IV
Complement Fixation Tests with Human Sera

Sera				Antigens (mouse brain)						
No	Diagnosis of disease by neutralization test	Interval between disease and bleeding for complement fixation test	Temperature of inactivation for 20 min	Louping ill	Lymphocytic choriomeningitis	Eastern equine encephalomyelitis	Western equine encephalomyelitis	St Louis encephalitis	Japanese B encephalitis	No antigen saline
1	Eastern equine encephalomyelitis	2 yrs	60		0	1 8*			0	0
2	" "	2 "	65	0	0	1 48		0		0
3	" "	2 "	65		0	1 8		0		0
4	" "	2 "	60		0	1 32		0		
5	" "	2 "	65		0	1 2		0		0
6	Western equine encephalomyelitis	3 "	65		0	0	1 8	0		
7	Western equine encephalomyelitis (?)	?	60		0	0	1 4	0		0
8	Western equine encephalomyelitis	2 yrs	60		0	0	1 1	0	0	
9	Louping ill	8 "	60	1 16	0	0			0	0
10	" "	4 "	60	1 4	0	0		0	0	0
11	Lymphocytic choriomeningitis	2 wks	60	0	1 4	0		0	0	0
12	" "	3 "	60	0	1 1	0		0	0	0
13	" "	2-4 wks	60		1 1		0	0		
14	St Louis encephalitis	4 mos	60			0	0	1 1		
15	" "	3 "	65		0	0		1 8		0
16	" "	3 "	60		0	0		1 1		0
17	" "	?	60				0	1 4		

* Highest dilution of serum giving a 2 plus or better reaction first dilution, 1 1 or 1 2

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CULTIVATION OF THE HOG CHOLERA VIRUS

By CARL TENBROECK M D

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research Princeton, New Jersey)

(Received for publication, July 7, 1941)

Although 9 years have passed since Hecke (1) reported the cultivation of the hog cholera virus, no confirmation of his work has yet appeared. This is probably in large part due to the fact that only swine can be used to demonstrate the virus and,—since the disease is so highly contagious,—to the necessity of keeping the inoculated animals in strict isolation.

Hecke used a number of swine tissues in his experiments. In hanging drop preparations containing choroid plexus or spleen he was able to demonstrate virus in the 15th passage. In flask cultures containing bone marrow in plasma plus Drew's solution virus was present in the 10th transfer while it was demonstrated in the 20th transfer of lymph node in plasma plus Drew's solution and in the 14th transfer containing spleen. Media containing kidney did not favor the increase of virus. There was great irregularity in the tests for virus in the various transfers and in all cases it was eventually lost. The least amount of culture tested was a 1:1000 dilution. Long incubation periods in the inoculated swine were not uncommon, suggesting either an attenuation of the virus or cross infection.

Hog cholera is an important disease and it seemed desirable to attempt to repeat and amplify the work already reported. Furthermore, since the virus is so highly specific for swine it was believed that its culture would be difficult, and that if it was successful, it might provide methods for the cultivation of some of the other specific viruses of man and animals. The results obtained are presented herewith.

Materials

A strain of virus used for vaccination in the central portion of the United States was obtained through the courtesy of Dr. Shope. It had probably been passed by inoculation through a series of swine and is highly virulent for them, causing a temperature and marked prostration in 2½ to 3 days after intramuscular injection. Swine that are killed after 3 or 4 days of fever show relatively few lesions: the lymph nodes are enlarged but seldom hemorrhagic, and a few hemorrhages are found under the capsule of the kidneys. If the animals are allowed to live for a longer period of time after injection, other characteristic lesions of hog cholera appear. The virus is neutralized by an anti hog cholera serum produced in the eastern United States by one of the large commercial houses.

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Materials

A strain of virus used for vaccination in the central portion of the United States was obtained through the courtesy of Dr. Shope. It had probably been passed by inoculation through a series of swine and is highly virulent for them, causing a temperature and marked prostration in 2½ to 3 days after intramuscular injection. Swine that are killed after 3 or 4 days of fever show relatively few lesions: the lymph nodes are enlarged but seldom hemorrhagic and a few hemorrhages are found under the capsule of the kidneys. If the animals are allowed to live for a longer period of time after injection, other characteristic lesions of hog cholera appear. The virus is neutralized by an anti-hog cholera serum produced in the eastern United States by one of the large commercial houses.

The swine used were raised on the grounds of the Department and were a cross between Chester Whites and Jersey Reds. The stock has not been vaccinated against hog cholera and has been free from disease. All inoculated animals were kept under strict isolation, one animal to a unit, and their temperatures were taken at least once daily. They were autopsied after they had had fever for 3 or more days.

Simms's (2) modification of Tyrode solution was used for tissue suspensions and dilutions were made in buffered salt solution. Fertile chicken eggs incubated 10 days and prepared by Burnet's technique (3) were used in some of the experiments.

Testicle tissue has been used because it is readily obtained without killing the animal and one pig will provide tissue for two experiments. Pigs weighing 60 to 90 lbs were etherized and one testicle was removed. This was immediately brought to the laboratory, flamed, and uncooked tissue removed from the interior, minced with fine scissors, and transferred to culture medium.

Methods and Results

Culture on Embryonated Eggs—When the work was started it seemed probable that some different technique would be necessary to propagate the virus, and it was decided to make use of the observation by Murphy (4) that mammalian tissue placed on the chorioallantoic membrane of embryonated eggs would live for a considerable period of time.

Virus, *z* e defibrinated blood from a pig bled 3 days after the development of temperature following inoculation with virus, plus Tyrode solution was mixed with minced swine testicle, and after standing about 10 minutes a small amount of tissue was deposited by means of a large calibre pipette on the chorioallantoic membrane of eggs containing 10-day embryos. The opening in the shell was covered with Scotch tape and the eggs were incubated at 37°C for 3 days. The eggs were then opened, and the membranes removed, ground, and suspended in Tyrode solution to approximately a 10 per cent suspension. (If the activity of the suspension was to be determined 10 per cent suspensions of the membranes were made.) This suspension was mixed with fresh minced swine testicle and eggs were again inoculated. This process was repeated every 3 days. Occasionally the eggs became contaminated and it was necessary to go back to material from earlier transfers that had been preserved in the refrigerator or dried. The amount of dilution at each transfer cannot be calculated, but it is at least 1/10 and probably much greater. The membrane suspensions were tested for virus at intervals and the results are given in Table I.

It will be seen that virus was demonstrated up to the 13th transfer, when the experiment was discontinued, and that a large amount was present since 1×10^{-7} cc of membrane suspension from the last transfer was sufficient to produce disease in a pig. The material from the 10th transfer, which is shown in the table to be highly infectious, was also injected subcutaneously into two pigs, each pig receiving at the same time, but in another area, 24 cc of anti-hog cholera serum. One pig received 1×10^{-1} cc and the other 1×10^{-4} cc of the membrane suspension. Neither pig showed a temperature and later both were

found to be immune to the original virus. Since our pigs have always been susceptible, this experiment shows that the passage virus was neutralized by anti hog cholera serum and that immunity followed the simultaneous injection of immune serum and virus.

There has been no evidence for the adaptation of the virus to the egg. On three different occasions during the course of the experiments membrane suspension that had been tested and found positive for virus was transferred to the chorioallantoic membrane of 10 day embryonated eggs without swine tissue. The eggs were incubated for 3 days, the membranes then suspended, and a fresh lot of eggs inoculated. After 3 days incubation the membranes from this lot of eggs were suspended and injected into swine. None of the swine developed a temperature and all were later shown to be susceptible to the

TABLE I

Tests for Hog Cholera Virus in Suspensions of Chorioallantoic Membranes on Which Were Deposited Minced Swine Testicle and Virus

Passage	Amount of 10 per cent membrane suspension that produced typical hog cholera	Remarks
	cc	
6	1×10^{-4}	Higher dilutions not tested
9	1×10^{-5}	10^{-6} cc not tested, 10^{-7} , 10^{-8} and 10^{-9} cc. negative
10	1×10^{-6} *	10^{-7} and 10^{-8} cc negative
12	1×10^{-6}	Higher dilutions not tested
13	1×10^{-6}	

* The protein nitrogen in the 10 per cent membrane suspension was 0.55 mg per cc.

virus. From eggs in the 12th transfer membranes and embryos were removed and a 10 per cent suspension was made of each. 1×10^{-1} cc of the embryo suspension produced acute hog cholera, whereas 1×10^{-4} cc did not. The membrane suspension was titrated at the same time and 1×10^{-6} cc caused acute hog cholera. The virus in the embryo suspension evidently was a contamination from the membranes, for if the virus had invaded the embryo we would expect that it would be present in a greater concentration than could be demonstrated.

Two tests were made to determine whether the passage virus would increase in chick embryos inoculated intravenously by the method described by Eichborn (5). The starting material was a proved infectious suspension from the 12th passage on the chorioallantoic membrane in the presence of swine testicle. In one experiment this material was injected intravenously into a set of eggs and after 24 hours a 10 per cent suspension of the embryos was made and 0.1 cc of a 10^{-2} dilution was injected into each egg in a second set. A 10 per cent suspension of the embryos in this second set was made after 24 hours incubation.

and 1 cc injected into a pig, with negative result. The experiment was repeated and the eggs were kept 3 days after inoculation instead of 24 hours. An embryo suspension from the 2nd passage failed to produce hog cholera when injected into a susceptible pig.

Search was made for a contaminating virus in material from the 11th transfer. Guinea pigs and rabbits given intracerebral and subcutaneous injections showed no rise in temperature and no evident illness. Mice injected intracerebrally lived for a month, and when they were finally disposed of were in good condition. Five mice, while under ether anesthesia, were inoculated intranasally with virus. Two were killed after 3 days and the remaining three after 20 days, and the lungs of all were found to be negative. The virus produced no lesions on the chorioallantoic membrane of eggs that contained 10-day embryos. These negative results make it extremely unlikely that a contaminating virus was present.

Flask Cultures—Along with the transfers in eggs as described above, cultures in Maitland's medium were carried

3 cc of Tyrode solution and 1 cc of sterile swine serum were combined in a 50 cc Erlenmeyer flask. To this was added a small amount of minced swine testicle and 0.5 cc of hog cholera virus. After 3 days incubation 0.5 cc of the first culture was transferred to freshly prepared medium. After 4 transfers the serum was omitted, the medium then consisting of 4.5 cc of Tyrode solution plus swine tissue and 0.5 cc of the previous culture. The dilution was then 1:10 at each transfer.

Virus was demonstrated in the cultures made at the 3rd, 6th, 9th, and 14th transfers. In the 14th transfer, 1×10^{-5} cc of culture produced acute and characteristic hog cholera.

Culture on Serum Agar—At about the time the above work was completed, reports of the culture of a number of viruses on serum agar plus minced embryo tissue began to appear (6-8). All these experiments were based on the study made by Zinsser and Schoenbach (9) and the application of these studies to the culture of typhus rickettsiae. A trial was made and it was found that the hog cholera virus also could be cultured in this way. The medium was the same as that described by Zinsser *et al.* (10) except that Simms's (2) modification was substituted for the regular Tyrode solution and serum from normal swine was used.

The serum was filtered through Seitz pads and stored in sterile containers. Two parts of this were mixed with 3 parts of double strength Tyrode solution, passed through a sterile Berkefeld N filter, and then mixed with 3 parts of sterile 3 per cent Difco agar in distilled water. The medium was transferred to Kolle flasks with a neck shaped to hold a No. 8 rubber stopper or to test tubes measuring 1.8×18 cm. These tubes, as well as the Kolle flasks, were closed with rubber stoppers after inoculation.

Minced swine testicle to which the inoculum had been added was spread over the surface of the solid serum agar. After 2 or usually, 3 days incubation at 37°C the tissue was washed off into 10 cc of buffered salt solution or Tyrode to each Kolle flask. This was usually diluted approximately 1:10 and passed through sterile Berkefeld filters before fresh inoculations were made.

The starting material was not the original hog cholera virus but was the material from the 13th passage on eggs. An additional 13 passages on the serum agar have been made and virus was demonstrated by swine inoculation as shown in Table II. The dilutions for swine inoculation were made in buffered salt solution without serum and the swine were immediately injected intramuscularly.

TABLE II

Tests for Hog Cholera Virus in Suspensions of Tissue from 3 Day Cultures on Swine Serum Agar

Passage	Material	Protein nitrogen per cc. of undiluted material	Least amount causing hog cholera
		mg	c
1	Not centrifuged	0.71	1×10^{-7}
9		—	1×10^{-6} *
10	Centrifuged and supernatant used	0.5	1×10^{-6} *
11		0.83	1×10^{-6} **
12		—	1×10^{-7}
13		0.57	1×10^{-8}

* Higher dilutions not tested

** A pig receiving 1×10^{-2} cc of this material intramuscularly in the hind leg and 24 cc. of anti hog cholera serum subcutaneously in the axillae showed no illness; i.e. the virus was neutralized.

SUMMARY

The work of Hecke on the cultivation of hog cholera virus was confirmed with ease. Virus was grown in the presence of fresh minced swine testicle in flasks containing Tyrode solution on the chorioallantoic membrane of embryonated eggs, and on the surface of swine serum agar. In flasks it was grown for 14 transfers, while on eggs it was grown for 13 transfers, followed by an equal number of transfers on agar, making 26 transfers in all. Only one strain of virus was used and we do not know whether all strains can be cultivated so readily or whether we were particularly fortunate in the selection of the strain used. Neither do we know whether swine testicle is better than other tissues for growth.

The cultured virus produces characteristic hog cholera when injected into swine, and its effect can be neutralized with commercial anti hog cholera serum. No evidence of attenuation of the virus was obtained, the last culture being

highly virulent when small amounts were injected. No evidence for the adaptation to the egg could be secured, since passages without swine testicle on the membrane or intravenously for 2 transfers resulted in a loss of the virus. No contaminating virus that might favor the cultivation could be detected by animal or egg inoculation.

Not only has the virus been cultivated but it has been demonstrated in large amounts in the culture. Four suspensions containing slightly over 0.5 mg. of protein nitrogen¹ produced typical hog cholera when 1×10^{-6} cc. was injected, and one suspension made in the same way was active in one-tenth this amount. Few titrations on what is commonly known as hog cholera virus, i. e. the serum from acutely ill pigs, are available. We made one such titration and produced a delayed disease with 1×10^{-5} cc. of infectious serum. It seems probable that the culture virus is more active than the commonly used virus and that its practical use in hog cholera vaccination and hyperimmunization would result in a considerable saving.

All of the methods used yielded active cultures, but the serum agar method is the one of choice since larger amounts of suspension can be obtained with less labor.

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¹ These determinations were kindly made by Dr. Roger M. Herrnott.

NEUTRALIZING ANTIBODIES IN HUMAN SERUM AFTER INFLUENZA A

THE LACK OF STRAIN SPECIFICITY IN THE IMMUNOLOGICAL RESPONSE

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Magill and Francis (1), by means of neutralization tests with specific immune animal serum, demonstrated that there were antigenic differences between certain strains of influenza A virus (2) This discovery was confirmed and considerably extended in subsequent investigations by these authors (3, 4) and by Smith and Andrewes (5) Magill and Sugg (6) reported that with human serum the titer of neutralizing antibodies may be dependent upon the virus strain used in the test. They also reported that during convalescence from influenza A neutralizing antibodies against several antigenically different strains of the virus were increased though not always to the same extent Taylor and Dreguss (7) obtained essentially similar results Andrewes, Smith, and Stuart Harris (8) presented evidence which indicated that influenza A might also result in the production of antibodies against swine influenza virus This finding was confirmed by Hare and Riehm (9)

The fact that there are quantitative, though not qualitative, antigenic differences between strains of influenza A virus would seriously complicate the study of immunity to influenza A if it were found that human beings produced a relatively strain specific antibody response to infection by a particular virus strain On the other hand, if, as seems probable from the results obtained by other investigators, there is a broad immunological response to influenza A in human beings, the importance to man of antigenic differences between strains of the virus diminishes in proportion to the breadth of the antibody response and its persistence in time

It is the purpose of this paper to present evidence which indicates that irrespective of the time which elapses after the onset of the disease, there is an almost complete lack of strain specificity in the immunological response which follows influenza A

Materials and Methods

Sera—Serum specimens were obtained from five adult patients who were confined in one ward of a state institution and who were ill with influenza A at approximately

the same time during February, 1939. The epidemic affecting this institution has been described previously (10). Specimens of serum were taken during the acute phase of illness from 2 to 4 days after the onset. Additional serum specimens were obtained approximately 10, 18, and 25 days, as well as 2, 3, 8, and 12 months, following the beginning of illness.

Viruses—Three antigenically different strains of influenza A virus were used.

(a) Strain 399 was isolated from the nasopharyngeal washings of one of the patients in this series (Case No. 1). After 9 serial passages in ferrets this strain was established in mice, in which species it was carried through 15 serial passages. Two suspensions of mouse lungs infected with this strain were employed. The 50 per cent mortality end points of these two suspensions were $10^{-4.5}$ and $10^{-5.4}$, respectively.

(b) The PR8 strain (11) after preliminary ferret passages had been carried through 332 serial passages in mice. The 50 per cent mortality end point of the suspension used was $10^{-6.5}$.

(c) The W S strain (12) was obtained through the courtesy of Dr. C. H. Andrews. Exact data as to the number of animal passages are not available. The 50 per cent mortality end point of the suspension used was $10^{-6.9}$.

One strain of swine influenza virus (No. 1976), which was obtained through the courtesy of Dr. R. E. Shope, was used. The suspension had a 50 per cent mortality end point of $10^{-3.7}$.

Standard suspensions of mouse lungs infected with each of the viruses mentioned above were prepared as described previously (13) and were stored in a low temperature cabinet (14) at -76°C .

Neutralization Tests—Neutralization tests with each of the virus strains indicated above were carried out on the various serum specimens from each of the five cases of influenza A. The technique of the neutralization test has been described previously (10). A constant quantity of the desired strain of virus was mixed with serial four-fold dilutions of serum. Serum dilution end points and virus titration end points were calculated by the 50 per cent mortality method of Reed and Muench (15).

Neutralizing Capacity—The neutralizing capacity of each serum against each strain of virus was calculated from the results of the neutralization tests by means of the equation

$$\log b = \log y - (a \log x) \quad (16)$$

EXPERIMENTAL

The neutralizing capacities of multiple serum specimens obtained from five cases of influenza A were determined against three antigenically different strains of influenza A virus and one strain of swine influenza virus. The results are presented in Table I. It will be seen that in the serum of Case 1, from whose nasopharyngeal washings Strain 399 was recovered, there occurred an increase in neutralizing capacity of more than $\log 2.60$ against the homologous strain of virus following infection. Closely similar increases in antibody level were also observed with both the PR8 and W S strains of influenza A virus, as well as with swine influenza virus.

It will also be noted that, with the single exception of Case 2, all five cases

TABLE I

The Neutralizing Capacity of Human Serum against Antigenically Different Strains of Influenza A Virus and Swine Influenza Virus at Intervals Following Influenza A

Case No	Days after onset of illness	Neutralizing capacity of serum against different viruses			
		Influenza A virus			Swine influenza virus
		PR 8	W.S	399	
		log	log	log	log
1 (Source of strain 399)	3	3.93 or <	4.12 or <	2.36 or <	3.19
	11	6.53	5.99	4.96	4.93
	18	6.75	5.99	4.96	4.78
	25	6.53	6.29	4.96	4.93
	60	5.67	5.99	4.53	—
	90	5.05	5.70	4.53	3.92
	240	4.94	4.99	3.67	4.32
	360	4.62	4.81	—	3.48
2	3	5.80	4.00	1.83 or <	5.97
	11	7.62	4.57	4.27	7.47
	18	7.40	4.57	4.27	6.99
	25	7.62	4.43	3.56	6.56
	60	6.53	4.00	3.41	6.56
	90	6.10	4.43	3.23	6.13
	240	5.67	4.57	2.70	5.83
	360	5.74	4.43	2.70	6.13
3	2	5.67	5.86	4.05 or <	5.83
	10	7.62	7.44	6.65	7.43
	17	7.40	7.59	6.65	7.28
	24	7.40	7.44	6.65	7.28
	59	6.53	6.95	5.93	6.31
	89	6.53	6.95	5.35	7.28
	239	6.23	6.29	>5.78	6.56
	360	5.80	6.72	4.92	6.40
4	2	4.62	4.12 or <	3.18 or <	4.83
	10	6.96	5.99	4.92	6.13
	17	6.53	5.86	4.92	6.13
	24	5.67	4.99	4.92	6.40
	59	5.51	4.99	4.92	5.54
	89	4.80	4.99	4.05	5.70
	239	4.80	4.81	4.05	5.54
	—	—	—	—	—
5	4	5.96	6.72	4.92	5.26
	12	7.70	7.74	6.21	6.71
	19	7.10	7.84	5.93	7.28
	26	8.12	7.32	—	—
	61	6.53	7.15	5.93	6.30
	241	5.80	6.72	4.92	6.13
	360	5.67	6.72	4.92	5.54
Mean all cases	2-4	5.20	4.96	3.27	5.02
	10-12	7.29	6.35	5.40	6.53
	17-19	7.16	6.37	5.35	6.59
	24-26	7.07	6.09	5.02	6.29
	59-61	6.15	5.81	4.94	6.18
	89-90	5.62	5.52	4.29	5.76
	239-241	5.49	5.48	4.23	5.68
	360	5.45	5.67	4.18	5.39

were found to have produced almost identical quantities of neutralizing antibodies against each of the virus strains used. Although in the sera from Case 2 similar concentrations of antibodies were found against Strain 399 and the PR8 strain of influenza A virus, as well as swine influenza virus, only a slight increase in antibodies was demonstrable against the WS strain.

The acute phase sera of the five patients varied considerably in their capacities to neutralize the different virus strains. Nevertheless, the acute

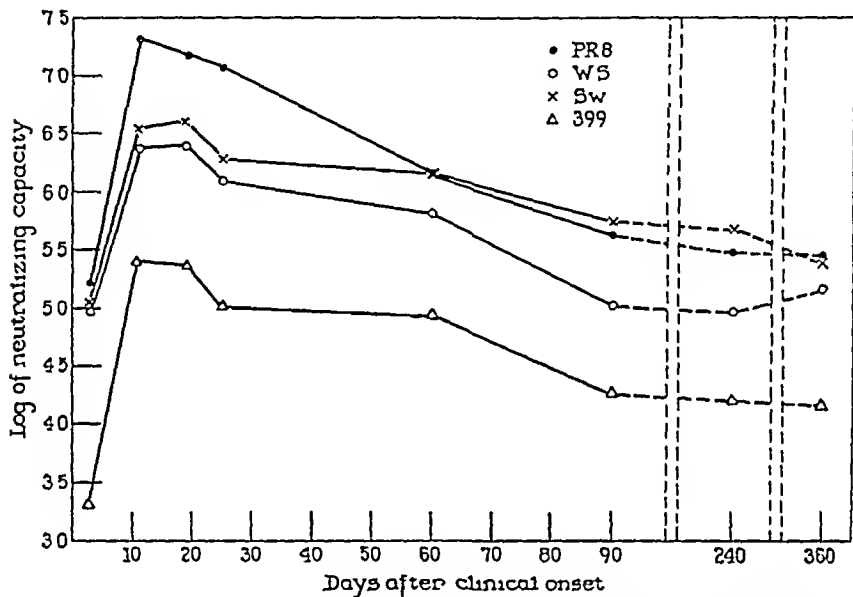


FIG. 1. Mean neutralizing capacities against three antigenically different strains of influenza A virus and swine influenza virus of serum obtained from five cases of influenza A at intervals after onset.

phase serum from each individual had a relatively constant neutralizing capacity against the PR8 and WS strains of influenza A virus and swine influenza virus. In the acute phase sera of all cases, however, the neutralizing capacities against Strain 399 were definitely lower than against the three other viruses.

In Fig. 1 the mean neutralizing capacities of the sera of the five cases against each of the four viruses are shown graphically. The mean neutralizing capacities have been plotted against the time after clinical onset at which the sera were obtained, and the experimental points have been connected by straight lines. It seems apparent that in these five cases of influenza A the observed increases in neutralizing antibody levels which followed infection

were as readily demonstrable when one strain of influenza A virus was used as when another antigenically different strain was employed. Furthermore, closely similar alterations in antibody levels were also encountered even when the antigenically distant swine influenza virus was used in the neutralization tests. It will be observed that the shape of the mean antibody level time curves shown in Fig 1 were almost identical irrespective of the strain of influenza A virus against which the sera were tested, and that even in the case of swine influenza virus, similar alterations in neutralizing antibodies with time were found.

Not only were the shapes of the antibody level curves similar, but under the conditions of these experiments it was found that the mean concentrations of neutralizing antibodies, at any interval studied, against the PR8 or W S strains of influenza A virus or even swine influenza virus were not very different. However, the mean antibody levels found when Strain 399 of influenza A virus was used in neutralization tests were at each interval definitely lower than those obtained with the other virus strains. It will be recalled that Strain 399 was recovered from the nasopharyngeal washings of Case 1. This strain of virus was not only causally related to the illness observed in Case 1 but undoubtedly was either antigenically very similar, if not identical, to those strains of virus which were associated etiologically with the disease encountered in the other four cases under study. It should be pointed out that Strain 399 had been carried through only a relatively small number of passages in mice and therefore, it may not have acquired full virulence for this species. Under these circumstances the 50 per cent mortality titration end point may not have given a fair indication of the quantity of virus present in the suspension. If a greater number of virus particles actually were contained in the suspension than might have been expected from the results of the titrations, it seems obvious that the determined neutralizing capacity of a serum would *apparently* be lower than otherwise.

It has been shown previously (17) that the increased antibody levels which follow influenza A rapidly decline. The results shown in Fig 1 present additional evidence in this regard. It will be seen that even between the 11th and the 25th day after onset of the disease there was with each of the strains of virus used an indication that a slight decrease in antibody concentration had occurred. The progressive lowering of neutralizing capacities continued during the 2nd month after onset and was somewhat more rapid during the 3rd month. Between the 3rd and the 12th months relatively little additional decrease in antibody level occurred. Three months after onset the mean neutralizing capacity against the four virus strains of the serum of the five cases was only log 0.65 higher than during the first days of their illness, and at 12 months it was but log 0.45 greater than in the acute phase of the disease.

ing even the simplest conditions favorable to the survival of the parasites *in vitro*. Bass and Johns (2) indicate that human malaria parasites must be anaerobic and that dextrose favors their survival. Manwell and Hewitt (15) stored *P. praecox* in sealed tubes, but Hewitt (11) states that *P. cathemerium* parasites seem to do better if spread in a very thin layer and hence under far from anaerobic conditions. Manwell and Hewitt (15) could not find any favorable effect of various concentrations of dextrose. Therefore it appeared desirable to undertake a somewhat systematic study of the effect of various *in vitro* conditions on the survival of malaria parasites.

Some care must be exercised in the interpretation of survival. For example, it is well known that many organisms can survive unfavorable conditions for a longer time at lower temperatures than at higher ones. This is true of malaria parasites too. *Plasmodium praecox* in citrated blood at 4-6°C retained its infectivity for 5 days (13), *P. gallinaceum* in defibrinated blood at 0-5°C remained infective for 21 days (9), and *P. knowlesi* and *P. mui* could be maintained in the frozen state at -76°C for 70 days or longer (6). The brief survival of *P. praecox* (15) and of *P. mui* (3) at 25°C but not at higher temperatures has already been noted. But certainly low temperature is not the kind of condition for survival which is of importance in attempts at the ultimate development of a culture method, for the malaria parasites of mammals and birds develop, in nature, at temperatures of 37.5 to 42 or 43°C. Very low temperatures are effective for the preservation of the parasites in a state of suspended animation for relatively long periods. But in the studies presented in this paper no effort has been made to obtain long preservation. The aim has been rather to study the comparative effects of a variety of agents and conditions on survival at temperatures which would be likely to bring about either development or rapid death of the organisms.

It is also necessary to establish suitable criteria for the determination of survival. Malaria parasites are typically non-motile. Something can be said as to their condition by observing their microscopic appearance in fresh and stained films. But, as Manwell and Hewitt (15) have already emphasized, the most objective and reliable criterion of survival is the ability of the parasites to infect a susceptible host. If the infectivity test is to be used for any extensive series of experiments, it is essential that the susceptible host be an experimental animal readily available in large numbers. It was largely for this reason that *Plasmodium lophurae* (5, 24, 25), a parasite capable of infecting chickens, was selected as the material for the experiments reported in the present paper. All of the experiments to be described were concerned with those stages only of the parasite which develop in the vertebrate host.

Methods

The Maintenance of Strains of Plasmodium lophurae—A strain of this parasite was obtained through the kindness of Dr. L. T. Coggeshall and has been maintained by

passage every 6 days in baby chicks. At each transfer, 0.05 ml of heparinized blood from the heart of a heavily infected chick is inoculated intracerebrally under ether anesthesia to each of a number of 2 day old chicks weighing about 25 gm. In such young chicks the intracerebral method of inoculation is fully as reliable in producing heavy infections which reach their peak on the 5th or 6th day as is the intravenous method used by Terzian (24) for 40 to 50 gm chicks. Moreover as judged by the minimum number of parasites required to produce an apparent infection 2 day old chicks inoculated intracerebrally are considerably more susceptible than approximately 1 week old chicks inoculated intravenously (see section on infectivity).

A strain of the parasite has also been maintained in ducklings 1 to 3 weeks old (32). At each transfer 0.2 to 0.5 ml of heparinized blood from the heart of a heavily infected duckling 6 days after its inoculation is injected intracardially into each of 2 or 3 fresh birds. The first duck inoculated with heavily infected blood from baby chicks showed only a light infection but since the third duck passage a heavy infection is regularly obtained which usually reaches its peak on the 5th day. By subinoculating on the 6th day it is possible to maintain a high parasite level which at the same time permits most of the hosts to survive.

Preparation of Parasite Material for the Survival Tests—From the heart of a 1 week old chick infected 5 days previously with *P. lophurae* it is possible to obtain 1 to 1.8 ml of blood representing at the most only 0.3 to 0.6 ml of red cells. About 10 ml of blood can regularly be obtained under sterile conditions from the heart of a chicken weighing about 300 gm. Hence it seemed desirable to use birds of this size as a source of parasites for the survival tests. One chicken could then yield enough material for a considerable series of preparations. However the chicken shows a marked age immunity toward *P. lophurae* (5, 22, 25). This difficulty has been overcome by the use of chickens injected intraperitoneally into the ventral right posterior portion of the peritoneal cavity with 5 ml. per 100 gm body weight of a 15 per cent by volume dilution containing 0.85 per cent sodium chloride of Higgins Fernald black writing ink (30). This amount of ink was administered on the day before inoculation with *P. lophurae* on the day of inoculation and for 2 or 3 days thereafter. Each chicken was inoculated intracardially with 0.8 to 0.9 ml of pooled heparinized blood from heavily infected baby chicks. 4 or 5 days after inoculation blood was taken aseptically from the heart of a chicken showing a sufficiently heavy infection into a syringe containing an appropriate volume of heparin sodium chloride solution (0.05 ml per ml of blood to be taken). The heparinized blood was centrifuged for 5 minutes the plasma was drawn off, and the cells were resuspended in the proper volume of a special balanced salt solution (see section below).

When parasites from ducks were desired a 2 or 3 week old duck on the 4th or 5th day of its infection was bled in the same manner as the ink treated chickens and the blood was treated in an identical way.

Media Used and Preparation of the Survival Tests—It appeared useful to have a suitable balanced salt solution for the washing and suspension of infected red cells as a diluent for various cell and organ extracts etc. Since malaria parasites live within the red cell and since the interior of the red cell has a very different composition from the serum which bathes it an attempt was made to construct a balanced salt solution based on available knowledge of the composition of red blood cells, and on certain general considerations. The composition of this solution to be designated hereafter

as solution K, is given in Table I, together with the composition of Tyrode's and Locke's solutions. Both of the latter roughly approach the composition of serum, and it will be noted that solution K has a much higher potassium content, a higher phosphate content, and a lower pH, in keeping with the properties of red cells as compared with serum (1, 16-18, 23). The potassium content of solution K is 330 mg per cent, as compared with a measured potassium content for goose erythrocytes of 230 mg per cent and for goose serum of 11.5 mg per cent (18). In the preparation of solution K, as well as of all other solutions, reagent salts, Pfannstiel dextrose, and double-distilled water were used. Such solutions were sterilized by filtration through a Berkefeld N filter. In some of the experiments it was convenient to have sterile

TABLE I
The Composition of Solution K, and of Tyrode's and Locke's Solutions

	Solution K	Concentration		
		Solution K	Tyrode's	Locke's
	gm per liter	mm per liter	mm per liter	mm per liter
NaCl	3.039	52.0	136.8	154.0
KCl	4.100	55.0	2.6	5.6
NaH ₂ PO ₄ · H ₂ O	0.690	5.0	0.4	—
K ₂ HPO ₄	2.613	15.0	—	—
NaHCO ₃	0.168	2.0	5.9	2.4
CaCl ₂	0.166	1.5	1.8	2.1
MgSO ₄ · 7H ₂ O	0.370	1.5	—	—
MgCl ₂ · 6H ₂ O	0.407	2.0	0.5	—
d Glucose	2.377	12.0	5.0	12.6
Total molarity		140.0	150.5	170.4
pH		7.2	7.3-8.0	7.4

concentrated solutions of the various constituents of solution K and to mix in sterile tubes the appropriate amounts of these and of sterile distilled water.

Heparin was the chief anticoagulant employed. A solution of 13.6 mg of Connaught highly purified heparin was prepared in 50 ml of 0.85 per cent sodium chloride solution. This was placed in small tubes and autoclaved, and the tubes were then capped with parafilm and stored in a refrigerator. In several experiments, solution K was modified so that it contained no calcium and, per liter, 5.0 gm of sodium citrate and only 2.046 gm of sodium chloride. This "citrate solution K" could then be used as the anticoagulant.

Early in the course of the work it became apparent that chicken red cell extracts greatly favored the survival of *P. lophurae*. These extracts were usually prepared in the following manner. Blood was taken aseptically from the heart of 1 to 2 month old chickens and was defibrinated by shaking with glass beads (or, more rarely, an anticoagulant was used). The blood was then centrifuged 5 minutes at a low speed and the serum (or plasma) drawn off. The cells were frozen by immersion of the

centrifuge tube in an alcohol dry ice mixture, and they were then allowed to thaw slowly in an incubator at 40°C. When they had thawed, the resulting dark red viscous liquid was mixed with an appropriate volume of diluent as solution K and the mixture was centrifuged at the high speed of an ordinary centrifuge for 1/2 to 1 hour. The clear red supernatant liquid was then drawn off and stored in a refrigerator at 8-9°C until used. Warburg (31) showed that a single freezing and thawing of avian red cells did not diminish their respiration and that even the nucleus free fluid showed a slight oxygen consumption. Red cell extracts were prepared 1 to 3 days before use. The term "dilute red cell extract" in the tables giving the experimental results refers to extracts in which the volume of diluent was at least twice the volume of red cells. For concentrated extracts a volume of diluent equal to that of the cells was used. With smaller volumes of diluent practically no nucleus-free supernatant liquid could be obtained since the frozen thawed cells after dilution and centrifugation had an appreciably greater volume than the untreated cells. For this reason, attempts to get very concentrated chicken erythrocyte extracts free from the cell nuclei have thus far failed. However in some of the work the frozen thawed red cells themselves either undiluted or slightly diluted were used.

Chick embryo extracts were prepared as follows. 10 day old embryos were removed aseptically, rinsed in a small dish of solution K, and their eyes removed. The embryos were then transferred to another sterile dish in which they were minced. The minced tissue was suspended in solution K, used in the proportion of 1.5 ml. per embryo, and the material was centrifuged 5 minutes at low speed. The opalescent supernatant liquid constituted the extract. Embryo extracts were prepared 1 day before use and were stored in a refrigerator.

The liver extract used for the experiments given in series 4, 5, 6, and 7 of Tables VIII and IX was made by extracting overnight 250 gm. of ground chicken livers (previously stored in the frozen state) in 50 ml. of citrate solution K at a pH of 6.4. The mixture was heated in a water bath to 65-70°C and held at this temperature for 10 minutes. It was then squeezed through gauze and filtered through paper to give a clear dark amber filtrate. The pH was adjusted to 6.7, the material was sterilized by Berkefeld filtration and was stored in the refrigerator. After a few days storage some sediment appeared. Other liver extracts were prepared in an essentially similar manner.

All extracts refer to chicken material, unless otherwise stated. Parasites from chicks were used only with chicken red cell extracts; parasites from ducks only with duck red cell extracts.

In the preparation of the survival tests the media to be tried were pipetted into appropriate sterile containers. The parasite suspension was then prepared as rapidly as possible and the media were inoculated with it. All the tests except a few preliminary ones were incubated at temperatures of from 39.5-42°C. Small Erlenmeyer flasks (Fig. 1) proved to be the simplest and most effective type of container. These 25 ml. pyrex flasks, previously cleaned with cleaning fluid, 10 per cent nitric acid, water and distilled water, and dried, were plugged with gauze lined cotton plugs. Over the mouth of each flask was placed a glass vial and the whole was sterilized by dry heat. Each such flask usually received 2 ml. of medium and 0.2 ml. of parasite suspension. The glass vial was then replaced and sealed to the side of the flask with

a ribbon of parafilm. This method of capping and sealing the flasks effectively prevented drying out of the contents and reduced the chances for contamination from the mouth of the flask. Most of the flasks were opened daily for examination, being held at room temperature only as long as absolutely necessary, and were then resealed with a fresh ribbon of parafilm. When a flow of gas through or over the medium was desired, a 75×25 cm vial or a 50 ml Erlenmeyer flask was used. This was equipped with a rubber stopper bearing gas entrance and exit tubes, each with a bulb stuffed with cotton. The gas was passed through distilled water and through a trap bottle before entering the experimental container.

In many of the experiments, one-third to one-half of the volume of medium was replaced daily with fresh corresponding medium which had been stored in the refrigerator. This was accomplished by gently tilting the flask and drawing off a portion of the supernatant liquid, being careful not to suck up the sediment of red cells and parasites. The quantity of liquid drawn off was measured and an equal quantity of fresh medium, first warmed to $38-40^{\circ}\text{C}$, was then added.

Determination of Survival —

Morphology of the Parasites —The study of dried films stained in the usual manner with Giemsa has proved to be more valuable than the examination of freshly prepared wet films. In most experiments, a Giemsa-stained dried film from each survival test was prepared and examined duly. When most of the parasites presented much the same appearance as in blood smears from an infected animal they were rated as good (G in the tables). When about half appeared degenerate, i.e. either the cytoplasm stained very darkly or the nuclei failed to stain, or both, the appearance was rated fair (F in the tables), and when most of the parasites appeared degenerate it was rated poor (P). It will be apparent from an examination of the results presented in the tables that the appearance of the parasites was not always a dependable indication as to their survival as judged by infectivity.

The Parasite Number —In some experiments, parasite counts were made per 1000 red cells on the original parasite suspension and on the smears prepared from the survival tests. Since an effort was made to count only normal appearing parasites, this second criterion of survival involves also the first one. However, in favorable preparations, during the first 2 or 3 days of incubation many of the parasites do appear normal and some useful information can be obtained from the counts. Hemocytometer counts showed that in these preparations the number of red cells per c. mm did not decrease significantly until the 3rd, and occasionally not until the 4th day. Hence the relative parasite number during the first 2 days is a dependable indication of the absolute parasite number.

Infectivity —Infectivity is a relative thing and has to be defined in terms of the actual operations used. For example, Boyd (4) and Demidowa (7) showed that with small numbers of *P. praecox* the length of the prepatent period varied inversely with the number of parasites inoculated. Boyd concluded that no apparent infection was produced by less than 1000 parasites, but Demidowa, by injecting a much larger number of birds and following them for a much longer time, found that even a single infected red cell could produce a visible infection (2 birds out of 70 injected). Obviously no comparisons can be made between an experiment in which conditions are such as to detect an infection produced by one or a few parasites surviving out of

millions originally present and an experiment in which no infection will be detected unless at least several hundred or several thousand infective parasites are present in the inoculum. Fulton (8) states that 10 ml of blood infected with the monkey parasite *P. knowlesi* and mixed with 0.1 ml of 50 per cent glucose solution was still infective 12 days later when it reached the United States from England. Temperature conditions during transit are not stated but in any case it is known that only a very few *P. knowlesi* injected into a *rhesus* monkey will produce a fatal infection. Since the original blood probably contained a very great number of parasites the percentage survival may actually have been almost infinitesimal.

For these reasons it was necessary to discover the minimum numbers of *P. lophurae* which would give infections apparent under the arbitrary standard conditions used to determine the infectivity of survival tests. These conditions were as follows. For each test of infectivity two 2 day old chicks were inoculated intracerebrally under ether anesthesia with a measured sample of material. On the 7th day and again on the 11th day after injection a Giemsa stained blood smear was prepared from each chick. Each smear was examined until parasites were found, but for a period not exceeding 5 minutes. If parasites were found in the 7 day smear the infectivity was designated ++ if they were not found in the 7 day smear but were found in the 11 day smear the infectivity was designated +, if they were not found in a 5 minute examination of both the 7 day and 11 day smears the infectivity was designated -.

The minimum numbers of parasites required to produce infection under these standard conditions were determined by making suitable dilutions in 0.85 per cent salt solution of freshly drawn heparinized or citrated blood on which a red cell and parasite count was made. Two chicks were inoculated intracerebrally with 0.03 ml of each dilution and blood smears were made and examined 7 days and again 11 days later. Seven experiments were performed using blood from infected chicks and 5 with blood from infected ducks. The results are given in Table II. The extent of variation was not great especially when one considers that only two chicks were used to test each dilution, and that the chicks were not of a genetically uniform stock. It can be said that on the average about 2000 parasites in chicken erythrocytes were required to produce an infection apparent on the 7th day, and about 200 parasites to produce one just apparent on the 11th day. When the parasites were present in duck erythrocytes, much larger numbers were required to produce infection in baby chicks as was to be expected. In 4 out of the 5 experiments about 100 000 was the minimum number giving an infection apparent on the 7th day. At least about 10 000 parasites had to be inoculated to produce an infection just apparent on the 11th day. It is interesting to note that Terzian (24) using 50 gm chicks inoculated intravenously could not detect any infections if an inoculum of less than 50 000 parasites (from chicks) was used even though smears were made for 18 days after inoculation. This of course explains his inability to demonstrate latent infections in recovered birds. By the use of the intracerebral technique with 2 day chicks it has been possible to demonstrate parasites in the blood of a rooster 6 months and 1 year after recovery from the initial infection.

Since most of the survival tests contained originally 50 000 to 100 000 parasites per c. mm. it is obvious that an infection apparent in 7 days after the inoculation of 50 c. mm. could have been produced by only 2000 parasites or 40 per c. mm. a survival

of less than 0.1 per cent. In the more recent experiments, suitable dilutions have been made to test for the survival of 40, 20, and 10 per cent of the organisms originally present. For example, in testing for 20 per cent survival of parasites in chick blood, the dilution was prepared in such a way that a dose of 20 c. mm. contained 10,000 parasites, on the basis of the original number present. If one or both of the chicks inoculated with this dose showed infection on the 7th day, it could be concluded that at least 20 per cent of the parasites were still infective. In a similar test using parasites in duck blood it would be necessary to have 500,000 parasites in the dose of 20 c. mm. In all the dilution tests, a dose of only 20 c. mm. was used, as this was never followed by leakage on withdrawal of the needle from the cranium.

TABLE II

The Minimum Numbers of Plasmodium lophurae Required to Give an Infection Apparent, in a 5 Minute Search of a Stained Blood Film, within 7 and within 11 Days after the Intracerebral Inoculation of 1 to 2 Day Old Chicks with 0.03 Ml. of Appropriate Dilutions in Saline of Freshly Drawn, Parasitized Blood

Source of infected blood	Experiment No.	Minimum No. of parasites giving infection in 7 days	Next lower No. tested	Minimum No. of parasites giving infection in 11 days	Next lower No. tested
Chicks	1	3,000	300	3,000	300
	2	2,000	1,000	200	—
	3	1,000	500	200	—
	4	600	300	250	—
	5	7,000	700	175	90
	6	850	213	213	—
	7	2,500	640	160	—
Ducks	1	150,000	15,000	15,000	1,500
	2	75,000	7,500	7,500	750
	3	170,000	87,000	11,000	—
	4	980,000	240,000	31,000	15,000
	5	150,000	74,000	37,000	18,000

In the earlier experiments it was found that infectivity generally remained high during the first 2 days of survival *in vitro*. For this reason, and in order to save on the number of chicks used, in the later experiments infectivity tests were not begun until the 3rd day.

Exflagellation of the Male Gametocytes—In order to observe this, a drop of material from the survival test was placed between slide and coverslip and allowed to stand at room temperature for 20–25 minutes. It was then examined for 5 minutes with a 4 mm. objective and 15× oculars, and the number of actively motile exflagellants seen was determined. When none was found in 5 minutes, the examination was usually prolonged to 10 minutes. It may be noted that 15–20 minutes is the time which usually elapses before exflagellation can be seen in a drop of blood removed from an infected host and held at room temperature. This criterion for survival is fully as objective as the criterion of infectivity and very much simpler to carry out.

Although it has thus far been used in only a few experiments it promises to yield in formation as reliable as that obtained from the infectivity tests. It is possible that conditions which are best for the survival and development of male gametocytes are not best for the asexual forms. This would be a most interesting fact to determine.

RESULTS

Effect of the Balanced Salt Solution Used for Diluting

Solution K was originally prepared with only 8 millimols of glucose per liter and a proportionately higher NaCl and KCl concentration (1 millimol of salt being considered as the osmotic equivalent of 2 millimols of glucose). In this form the solution was tested against Locke's and against Tyrode's solution by mixing a little infected blood with each solution in a small test tube and later observing the appearance and infectivity of the material. For example in one test, on the 2nd day of incubation at 36°C, the parasites in Locke's solution presented a poor appearance and a — infectivity, while those in solution K had a fair appearance and a + infectivity. Again, in another test in Locke's solution on the 2nd day the appearance was poor and the infectivity +, while in solution K the appearance was fair and the infectivity ++. Similar results were obtained in comparing solution K with Tyrode's solution.

In another experiment, the same amount of the same parasite suspension was inoculated to each of 6 tubes containing the following materials: (1) Locke's solution, (2) Locke's solution with an anti chick red cell rabbit serum, (3) the same as (2) plus complement (guinea pig serum), (4) solution K, (5) solution K with the anti chick red cell rabbit serum, and (6) the same as (5) plus complement. After 1 day at 36°C the infectivity was ++ in tubes 1 and 4, in tubes 2 and 5 there was partial hemolysis and the infectivity of tube 2 was — while that of tube 5 was ++, in tubes 3 and 6 there was apparently complete hemolysis, and the infectivity of tube 3 was — while that of tube 6 was +.

Effect of the Degree of Aeration

Some typical results obtained on this matter are summarized in Table III. The exposure of *P. lophurae* to air has evidently a very favorable effect on their survival *in vitro*. This was always the case, whether the extent of aeration was increased by spreading the parasites in a thin layer, by passing a current of air, or by suspending them in a semisolid gel. In preparations 2 and 3 of Table III, the melted 2 per cent agar was brought to a temperature of 45°C and added to the tubes after all the other ingredients had been put in. The contents of the tubes were stirred vigorously and the tubes were plunged into a beaker of ice water for a minute or two. This rapidly set the medium into a semisolid gel in which the parasites were uniformly distributed. It is also evident from Table III that a high oxygen tension as in almost pure O₂ or 95 per cent O and 5 per cent CO₂, was detrimental. In several other experi-

TABLE III

Effects of Aeration and High Oxygen Tension on the Survival of Plasmodium lophurae at 39.5-40°C

No	Type of preparation	Appearance* and infectivity** after days				
		1	2	3	4	5
1a	Dilute frozen thawed red cell extract in solution K 1 ml in small test tube + 0.2 ml parasite suspension Height of liquid 15 mm		F++	P-		
1b	Same medium as for 1a 2.5 ml in 25 ml Erlenmeyer flask + 0.5 ml same parasite suspension Height of liquid 4 mm		G++	G++		
2	4 ml dilute heat hemolyzed red cell extract in solution K + 0.5 ml parasite suspension first partly hemolyzed with hypotonic red cell extract and then resuspended in isotonic red cell extract + 0.5 ml 2% agar in isotonic NaCl solution Column in tube 4 cm high	G+† G+ F-	F+ F- F-			
3	5.6 ml dilute heat-hemolyzed red cell extract + 1.6 ml parasite suspension + 0.8 ml 2% agar in isotonic NaCl solution Column in tube 4 cm high		G++† F++ F+			
4a	5.2 ml dilute frozen thawed red cell extract in solution K + 0.8 ml parasite suspension in vial to depth of 17 mm Current of air bubbled through	G++	G++	F++	F+	P+
4b	Same as 4a but no air bubbled through	G++	F+	P-	P-	
5a	2.5 ml frozen thawed red cell suspension + 1 ml embryo extract + 0.3 ml liver extract + 1.2 ml solution K In vial with air bubbled through	G	F+	F+		
5b	Same as 5a but no air bubbled through	G	F+	P-		
6a	1.5 ml embryo extract + 0.5 ml liver extract + 1.5 ml 4% agar to make solid gel in a 50 ml Erlenmeyer flask. Added 1 ml frozen thawed red cells + 0.4 ml parasite suspension Stream of CO ₂ -free air passed over surface		G	G++		
6b	Same as 6a but oxygen passed over		G	F-		
7a	3.1 ml dilute red cell extract in solution K + 0.4 ml embryo extract + 0.1 ml liver extract + 0.4 ml parasite suspension in 50 ml Erlenmeyer flask. Mixture of 95% O ₂ , 5% CO ₂ passed over surface	G	G++	F-	P-	
7b	Same as 7a but mixture of 95% air, 5% CO ₂ passed over surface	G	G++	G++	F++	

* In this and all subsequent tables,
G = all or most of parasites appear normal,
F = some of the parasites appear normal,
P = few or none appear normal

** In this and all subsequent tables,

-- = infection apparent 7 days after inoculation of 2 day old chick intracerebrally with 0.05 ml. of material,

+ = infection apparent 11 days after inoculation, but not 7 days after,

- = no infection after 11 days

† In Experiments 2 and 3, samples were taken from the top, middle, and bottom of the column of semisolid gel in the tube Results are given in that order

ments, no difference could be detected between survival when air was passed through and survival when a mixture of 50 per cent air and 50 per cent oxygen was passed through

The Effects of Glucose, Glycogen, and Glutathione

Bass and Johns (2) found that glucose and maltose, but not a series of other sugars and related compounds, favored the survival of *P. falciparum*. Since then, no other detailed experimental work on the problem has been reported. Fulton (8), however, measured the effect of various sugars on the rate of respiration of suspensions of *P. knowlesi*. Only glucose, levulose, maltose, mannose, and glycerol produced an increase in oxygen uptake as compared with that of parasites suspended in sugar free medium.

The results of several typical experiments on the effects of added glucose on the survival of *P. lophurae* are given in Table IV. In all the preparations red cell extract and embryo extract were present so that carbohydrate substrates were available apart from the glucose added, or not, with the solution K used in making the red cell extract. It is apparent, nevertheless, that added glucose favored survival (3 a, b, 7 a, b), that the presence of 12 millimols of glucose per liter was more favorable than 8 millimols per liter (2 a, b), and that a still higher concentration of glucose, 24 millimols per liter, was harmful (1 a, b, 3 b, c). This latter effect may have been due to a toxic impurity in the glucose.

Glycogen, at concentrations by weight approximately equivalent to the favorable concentrations of glucose, could replace glucose and, in fact, was even better than glucose in most of the tests conducted (4 a, b, c, 5 a, b, of Table IV). It is interesting to note in this connection that glycogen did not stimulate the respiration of the suspensions of *P. knowlesi* (8).

The results with the addition of glucose and glycogen showed up best when fairly dilute red cell extracts were used. With such extracts a consistently favorable effect of added glutathione could also be demonstrated (5 a, c, 6 a, b, of Table IV).

The Effects of Renewing the Medium and of the Density of Parasites and Red Cells

Repeated trials showed that other conditions being the same, survival for several days longer could be obtained if one-third to one-half of the medium was drawn off daily and replaced by fresh medium which had been stored in the refrigerator. When this was done, the contents of the flasks would retain a rather bright red color, instead of getting decidedly brownish as in flasks in which the red cell extract medium was not renewed. It would probably be even better to renew all of the medium daily, but with the flat shallow layers of liquid which had to be used, it was impossible to draw off more than half of the medium and still avoid sucking up some of the sedimented red cells.

TABLE IV

Effects of Glucose, Glycogen, and Glutathione on the Survival of Plasmodium lophurae

(All preparations held in 25 ml Erlenmeyer flasks at temperatures of 39.5–40°C, except for 7 a and 7 b, which were kept at 41–41.5°C)

No	Type of preparation	Appearance and infectivity* after days					
		1	2	3	4	5	6
1 a	1.9 ml dilute red cell extract in solution K but with 8 mM glucose per liter, NaCl and KCl raised proportionately, + 0.1 ml embryo extract + 0.15 ml parasite suspension		G++	F+	P-		
1 b	Same as 1 a, but 24 mM glucose per liter, NaCl and KCl lowered proportionately		G-	P	P		
2 a	1.8 ml dilute red cell extract in solution K but with 8 mM glucose per liter, NaCl and KCl raised proportionately, + 0.2 ml embryo extract + 0.2 ml parasite suspension		G++	F++	P-		
2 b	Same as 2 a but standard solution K with 12 mM glucose per liter		G++	F++	F++		
3 a	1.84 ml dilute red cell extract in solution K with glucose omitted, NaCl and KCl raised proportionately + 0.16 ml embryo extract + 0.2 ml parasite suspension	F	F++	P-			
3 b	Same as 3 a but standard solution K with 12 mM glucose per liter	G	F++	F+	P-		
3 c	Same as 3 a but with 24 mM glucose per liter, NaCl and KCl lowered proportionately	G	F-	P-			
4 a	1.8 ml dilute red cell extract in solution K with glucose omitted, NaCl and KCl raised proportionately + 0.16 ml embryo extract + 0.04 ml liver extract + 0.2 ml parasite suspension		G++	F++	F-	P	
4 b	Same as 4 a but using solution K with glucose replaced by 0.34% glycogen, NaCl and KCl raised proportionately		G++	G++	F++	F-	
4 c	Same as 4 b, but 0.18% glycogen		G++	G++	F++	F-	
5 a†	1.8 ml dilute red cell extract in solution K + 0.16 ml embryo extract + 0.04 ml liver extract + 0.2 ml parasite suspension		G	G++	F+	P-	

TABLE IV—*Concluded*

No	Type of preparation	Appearance and infectivity after days					
		1	2	3	4	5	6
5 b†	Same as 5 a but glucose replaced by 0.18% glycogen NaCl and KCl raised proportionately		G	G++	F++	P+	
5 c†	Same as 5 a + 0.2% glutathione		G	G++	F++	P++	
6 a†	1.84 ml dilute red cell extract in 1 part serum + 3 parts solution K with glucose replaced by 0.2% glycogen NaCl and KCl raised proportionately + 0.16 ml embryo extract + 0.2 ml parasite suspension		G	G++	F++	P+	
6 b†	Same as 6 a + 0.1% glutathione		G	F++	F++	P++	
7 a†	1.64 ml dilute red cell extract in solution K with glucose omitted NaCl and KCl raised proportionately + 0.16 ml embryo extract + 0.2 ml parasite suspension	G	G	F++	F+	P+	P+
7 b†	Same as 7 a but with standard solution K with 12 mg glucose per liter	G	G	F++	F++	P++	P+

* See footnotes Table III

† In these flasks about 0.8 cc of the medium was replaced daily with medium stored in the refrigerator

In several tests, the contents of a flask were transferred to a centrifuge tube and centrifuged 5 minutes. The liquid was then drawn off and the sediment suspended in fresh medium. This procedure, perhaps because of the centrifuging, resulted in the rapid death of the parasites.

It was also observed in various experiments that poor survival was obtained if the parasite suspension used had a very high absolute parasite number. An experiment designed to test this and the preceding point is illustrated in Table V. Of the two flasks which each contained 2×10^5 parasites per c. mm. the medium was partially renewed daily in one, which gave a ++ infectivity on the 5th day, but not in the other, which already on the 3rd day gave a - infectivity. Of the other four flasks, in two the parasites only were decreased, the density of red cells being kept about constant, and in two both the red cells and parasites were decreased. The medium was not renewed in any of these flasks. It is evident that the smallest density of parasites gave the longest survival in both cases, the effect of total red cell concentrations being relatively slight. However, the best infectivity of all, on the 5th day, was obtained in

the flask which had the highest parasite density but had received fresh medium daily. The relative numbers of normal looking parasites gave results somewhat parallel to those based on infectivity. Especially noteworthy are the figures for flasks 1, 4, and 6 on the 3rd day.

TABLE V

Effects of the Density of Parasites and Red Blood Cells and of Renewal of the Medium on the Survival of Plasmodium lophurae

(All preparations held in 25 ml Erlenmeyer flasks at 39.5–40°C. Each flask contained 1.8 ml of the following medium. One part of frozen thawed red cells extracted with 3 parts solution K and nuclei removed by centrifugation. To 15 ml of this extract added 1 ml embryo extract. In flask No. 1 0.8 to 1.0 ml of medium was replaced daily with medium stored in the refrigerator.)

Flask No	Parasite suspension added ml	Uninfected red cell suspension added ml	Parasites per c mm	Red cells per c mm	Parasites per 1000 red cells after days				Appearance and infectivity * after days					
					0	1	2	3	1	2	3	4	5	6
1	0.2	0	2×10^5	3.2×10^5	674	522	336	92	G	G	Γ++	P++	P++	
2	0.1	0.1	1×10^5	3.5×10^5	286	170	208		G	G	Γ++	Γ+		
3	0.05	0.15	0.5×10^5	3.6×10^5	140	102	90		G	G	F++	Γ++	P+	—
4	0.2	0	2×10^5	3.2×10^5	674	468	330	36	G	G	F—	P—		
5	0.1	0	1.1×10^5	1.7×10^5	674	468	408		G	G	Γ++	F++	P—	
6	0.05	0	0.6×10^5	0.9×10^5	674	458	428	100	G	G	Γ++	Γ++	Γ+	—

* See first footnote, Table III

Effects of Serum, Plasma, and Embryo Extract

In a series of experiments using relatively concentrated red cell extracts prepared in varying concentrations of serum and citrated plasma, observations were made on exflagellation as well as on infectivity and relative parasite numbers. The results of some of these experiments are summarized in Tables VI to IX. All the preparations were held in 25 ml Erlenmeyer flasks at 41.5–42°C. Except for preparation 1c, each flask received originally 2 ml of medium and 0.2 ml of parasite suspension. Every day 0.6 to 1.0 ml of medium in each flask was replaced by corresponding medium which had been stored in the refrigerator. It will be noted that the original density of parasites was held at about 30,000 to 50,000 per c mm for suspensions from chicks, and at about 60,000 to 80,000 per c mm for suspensions from ducks. Table VI shows that survival was very short in citrated chicken plasma (1d) but was prolonged if the plasma was diluted somewhat and contained red cell extract (1c). Chicken serum alone gave a + infectivity even on the 6th day (1b),

while the infectivity of the preparation (1 a) with diluted serum and red cell extract was gone after the 5th day. However, the latter preparation had a higher infectivity on the 4th day than did the serum alone and also gave

TABLE VI

Effects of Serum Plasma and Red Cell Extract on Survival of Plasmodium lophurae

No	Medium	Parasites per c. mm	Red cells per c. mm	Infectivity* after days				Exflagel- lant† after days		Parasites per 1000 red cells after days			
				3	4	5	6	5	6	0	1	2	3
1 a	Red cell extract—8 ml. frozen thawed cells in 6 ml. citrate solution K + 2 ml. serum	5×10^5	2.3×10^5	20% ++	10% ++	+	—		0	226	206	146	70
1 b	Serum—same as used for 1 a	5×10^5	2.3×10^5	20% ++	10% +	+	+	1	0	226	157	78	36
1 c	Red cell extract—8 ml. frozen thawed cells in 6 ml. citrate solution K + 3 ml. citrated plasma (1 part citrate solu- tion K to 3 parts blood)	5×10^5	2.3×10^5	20% ++	10% ++	—	—		0	226	230	168	74
1 d	Citrated plasma—same as used for 1 c	5×10^5	2.3×10^5	10% —	—	—	—			226	172	48	
1 e	Mixed 1 ml. of red cell-embryo extract (8 ml. frozen-thawed cells in 14 ml. solution K— to 10 ml. extract added 1 ml. embryo extract) with para- site suspension and 1 ml. of heparinized plasma. Al- lowed mixture to clot. Then added 0.8 ml. of the red cell embryo extract. Clot ground up for test on 5th day	7×10^5	2.9×10^5	—		++		10		240		226	100

* 20% ++ signifies an infection apparent 7 days after the inoculation of the chick with a dilution such that the chick received on the basis of the number of parasites originally present a total dose of 10 000 parasites if contained in chick red cells or of 500 000 parasites if contained in duck red cells and indicates that at least 20% of the parasites originally present were still alive. (See section on infectivity under methods.) 20%+ signifies under similar conditions, an infection apparent after 11 but not after 7 days, and 20%— no infection after 11 days. 10% and 40% with ++ + or — have a similar significance except that the dose would be respectively 20 000 and 5 000 parasites in chick cells and 1 000 000 and 250 000 parasites in duck cells. ++ + or — alone have the same significance as in Table III. In 1 a b c on the 4th day inoculations were made with undiluted material as well as with a dilution testing for 10 per cent survival.

† The number of exflagellants counted in a 5 minute examination of a fresh preparation is given except that, if none were found on the 5th and 6th days the time of examination was extended to 10 minutes.

consistently higher parasite counts during the first 3 days. Preparation 1 e is of special interest. The parasite suspension was here included in a clot consisting of equal parts red cell-embryo extract and heparinized plasma. The

TABLE VII

Effects of the Concentration of Plasma, and of Chick Embryo and Yeast Extracts, on Survival of Plasmodium lophurae

No	Medium	Parasites per c mm	Red cells per c mm	Infectivity* after days				Exflagellants† after days						Parasites per 1000 red cells after days			
				3	4	5	6	1	2	3	4	5	6	0	1	2	3
2 a	Red cell extract—6 ml frozen thawed cells in 8 ml citrated plasma (1 part citrate solution K to 3 parts blood)	43×10^5	2×10^5	20% ++	10% +	+	—	1	2	2	1	0		220	232	182	116
2 b	Red cell extract using same cells and plasma as for 2 a—6 ml frozen thawed cells in 5 ml citrated plasma + 2.6 ml citrate solution K	43×10^5	2×10^5	20% ++	10% ++	+	++	3	1	4	4	0		220	222	192	98
2 c	Red cell extract—9 ml frozen thawed cells in 5 ml citrated plasma + 10 ml citrate solution K	43×10^5	2×10^5	20% ++	10% +	+	+	2	1	0	0			220	216	170	86
2 d	Same as 2 c but with 10% by volume of chick embryo extract in citrate solution K	43×10^5	2×10^5	20% ++	10% ++	++	+	1	5	1	0			220	218	180	126
2 e	Red cell extract—11 ml frozen thawed cells in 5 ml citrated plasma + 10 ml citrate solution K. To 5 ml extract added 0.6 ml yeast extract	43×10^5	2×10^5	20% ++	10% +	+	—	0	3	1	0			220	234	156	116
2 f	Same as 2 e but to 5 ml red cell extract added 0.1 ml yeast extract	43×10^5	2×10^5	20% ++	10% ++	++	+	3	0	1	0			220	236	168	126
2 g	Red cell extract similar to that for 2 e but using blood from other chicks. To 5 ml extract added 0.01 ml yeast extract	43×10^5	2×10^5	20% ++	10% +	++	+	2	3	0	0			220	214	174	88
3 a	Duck red cell extract—4 ml frozen thawed cells in 7 ml citrated plasma (1 part citrate solution K to 3 parts duck blood). Parasites from duck	6×10^5	1.2×10^5	20% +	++	+	—	3	16	8	14	2	0	570	524	346	
3 b	Duck red cell extract using same cells and plasma as for 3 a—4 ml frozen thawed cells in 4.7 ml citrated plasma + 2.3 ml citrate solution K	6×10^5	1.2×10^5	20% ++	+	+	—	15	30	8	7	6	0	570	500	340	
3 c	Duck red cell extract—12 ml frozen thawed cells in 5 ml citrated plasma + 10 ml citrate solution K.	6×10^5	1.2×10^5	20% ++	—	—		15	5	1	0			570	474	306	
3 d	Same as 3 c but with 10% by volume of chick embryo extract in citrate solution K	6×10^5	1.2×10^5	20% +	+	—		6	7	7	0	0		570	516	296	

* † See corresponding footnotes for Table VI

clot was covered with liquid red cell-embryo extract which was renewed daily. During the first 4 days small samples were removed from the clot and used to make smears. On the 5th day the clot was ground up and tested for infec-

tivity This proved to be ++ A large number of exflagellants was observed on this day This preparation gave high relative parasite counts, the count on the 2nd day being essentially the same as in the original parasite suspension Similar preparations have consistently given similarly good results but entirely liquid preparations are much easier to handle and are preferable for comparative tests of various ingredients of the medium

TABLE VIII

Effects of Different Concentrations of Liver Extract on Survival of Plasmodium lophurac

No	Medium	Parasites per c mm	Red cells per c mm	Infectivity after days			Exflagel lantst after days					Parasites per 1000 red cells after days			
				3	4	5	6	1	2	3	4	5	0	1	2
4 a	Red cell extract—17 ml frozen thawed cells in 27 ml citrated plasma + 13.5 ml citrate solution K. To 57 ml extract added 0.3 ml citrate solution K.	35×10^6	1.9×10^8	2000 +	1000 —	—	—	3	4	6	2	0	194	176	92
4 b	57 ml same red cell extract + 0.29 ml citrate solution K + 0.01 ml liver extract in citrate solution K.	35×10^6	1.9×10^8	2000 +	—	—	—	9	18	12	2	0	194	162	134
4 c	57 ml same red cell extract + 0.27 ml citrate solution K + 0.03 ml liver extract	35×10^6	1.9×10^8	2000 +	1000 +	—	—	6	12	2	2	0	194	142	116
4 d	57 ml same red cell extract + 0.2 ml citrate solution K + 0.1 ml liver extract	35×10^6	1.9×10^8	2000 ++	1000 —	—	—	3	1	0	0	—	194	124	140
4 e	57 ml same red cell extract + 0.3 ml liver extr ct	35×10^6	1.9×10^8	2000 +	1000 —	—	—	0	0	0	0	—	194	—	—
4 f	51 ml same red cell extract + 0.6 ml embryo extract + 0.29 ml citrate solution K + 0.01 ml liver extract	35×10^6	1.9×10^8	2000 +	1000 —	+	—	1	32	8	0	0	194	176	106
5 a	Duck red cell extract—3 ml frozen thawed cells in 30 ml citrated duck plasma + 15 ml citrate solution K. To 57 ml extract added 0.3 ml citrate solution K. Para sites from duck	87×10^6	1.3×10^8	++	++	+	+	2	6	2	0	—	720	608	404
5 b	51 ml same red cell extract + 0.6 ml chick embryo extract + 0.3 ml citrate solution K	87×10^6	1.3×10^8	++	+	+	+	8	5	4	2	0	720	636	468
5 c	Same as 5 b but 0.29 ml citrate solution K and 0.01 ml liver extract	87×10^6	1.3×10^8	++	+	+	+	1	15	8	0	0	720	682	448
5 d	Same as 5 b but 0.25 ml citrate solution K and 0.05 ml liver extract	87×10^6	1.3×10^8	++	—	—	—	7	3	1	—	—	720	—	—
5 e	Same as 5 b but 0.3 ml liver extract	87×10^6	1.3×10^8	—	—	—	—	5	0	0	—	—	720	—	—

* † See corresponding footnotes for Table VI

Table VII, 2 a-c, shows that red cell extract prepared in about equal parts of plasma and citrate solution K (b) was better than a similar extract in whole citrated plasma (a — one part citrate solution K to 3 parts plasma) or an extract in one part of plasma to 3 parts of citrate solution K (c) The results on infectivity, exflagellation, and parasite number are all in fair agreement However, although the infectivity of preparation 2 c was slightly better than

that of 2 *a*, the reverse was true of the exflagellation. Preparations 3 *a-c* were similar to 2 *a-c* but with duck material. Here the highest plasma concentration proved to be as good as, or perhaps slightly better than, the intermediate concentration, while the lowest concentration was definitely worse in all respects.

Preparations 2 *c, d* illustrate the consistently favorable effect of the addition of chick embryo extract to the medium at a concentration not exceeding 10 per cent by volume. Higher concentrations were deleterious. The effect is also shown by preparations 3 *c, d*, and by 4 *b, f* of Table VIII. Preparations

TABLE IX

The Survival of Plasmodium lophurae in Concentrated Red Cell Extracts with and without Liver Extract

No	Medium	Parasites per c mm	Red cells per c mm	Infectivity* after days				Exflagellants† after days						Parasites per 1000 red cells after days		
				3	4	5	6	1	2	3	4	5	6	0	1	2
6 <i>a</i>	Red cell extract—14 ml frozen thawed cells in 7 ml serum + 7 ml solution K. To 6 ml extract containing some of the nuclear residue added 0.6 ml embryo extract.	3×10^5	2.4×10^5	40% +	20% +	++	+	4	10	9	17	0	0	134		
6 <i>b</i> ‡	Red cell extract—12 ml frozen thawed cells in 6 ml serum + 6 ml solution K. To 6 ml extract containing a light suspension of free nuclei added 0.6 ml embryo extract + 0.01 ml liver extract.	3×10^5	2.4×10^5	40% ++	20% ++	+		6	2	5	0	0		134	178	148
7 <i>a</i>	Duck red cell extract—14 ml frozen thawed cells in 7.5 ml duck serum + 7.5 ml solution K. To 6 ml extract containing some of nuclear residue added 0.6 ml chick embryo extract. Parasites from duck.	8×10^5	1.5×10^5	20% ++	10% ++	++	—	18	13	12	3	1	0	568		
7 <i>b</i>	Duck red cell extract—12 ml frozen thawed cells in 6 ml duck serum + 6 ml solution K. To 7 ml clear extract added 0.7 ml chick embryo extract + 0.01 ml liver extract.	8×10^5	1.5×10^5	20% ++	10% +	+	—	26	12	17	5	15	0	568	604	532

* † See corresponding footnotes for Table VI

‡ This preparation was accidentally contaminated on the 5th day

2 *e, f, g* show the effects of a yeast extract prepared by boiling 2.5 gm of dried Harris brewers' yeast in 25 ml of citrate solution K, filtering through paper, and sterilizing by Berkefeld filtration. Of the three concentrations tried, the intermediate one was slightly better than the others.

The effects of the addition of chicken liver extract using chick and duck material are shown by series 4 *a-e* and 5 *b-c* respectively of Table VIII. In

these experiments equal parts of plasma and citrate solution K were used in the preparation of dilute red cell extracts, and the survival was not as good as in the preceding series, employing more concentrated red cell extracts. In the chick series, the lowest concentration of liver extract tried (4 b) definitely enhanced the exflagellation. The chicks used for the 4th day infectivity test on this preparation both died before results could be obtained from them, but the preparation (4 c) with the next higher concentration of liver extract showed a better infectivity than the preparation without liver extract. Still higher concentrations of liver extract were toxic. With the duck material the lowest concentration of liver extract did slightly enhance the exflagellation on the 2nd and 3rd days, but no effect on infectivity was observed. The higher concentrations were again toxic.

Preparations 6 a, b, and 7 a, b of Table IX show the results of tests with as concentrated red cell extracts made in equal parts of serum and solution K, as could conveniently be prepared in the necessary amount. Preparations 6 a and 7 a contained in the extract a considerable amount of the nuclear residue and the presence of these numerous free nuclei precluded accurate parasite counts. Preparation 6 b contained some free nuclei and preparation 7 b very few. In preparation 6 b, 40 per cent or more of the original parasites were still alive on the 3rd day and 20 per cent or more on the 4th day. The parasite number increased by a significant amount after 1 day's incubation, but by the 2nd day it had dropped back to about the original figure. No significant decrease in total red cell count was observed in this preparation until the 4th day. In preparation 7 b the parasite number maintained itself for the first 2 days, with a possible increase on the 1st day. In this preparation, the red cell count went down after the 2nd day. Duck red cells have typically proved to be less resistant to the *in vitro* conditions than have chick red cells. It is worth noting that in preparation 7 b as many as 15 exflagellants were found in 5 minutes in a sample taken after 5 days of life *in vitro* at a temperature of almost 42°C.

DISCUSSION

Only two questions need be discussed here. Were the various effects which were observed direct effects of the environmental conditions on the malaria parasites or were they indirect effects *via* the red cells in which the parasites were contained? Did the parasites which survived in these experiments continue to develop while surviving? To the first question no answer can be given. Since there is as yet no way of maintaining the parasites outside of red cells, the effects of environmental conditions on the two cannot be readily separated. It can be said that many normal appearing erythrocytes were still present in preparations in which the parasites had died out. Also, dextrose appears to have no effect on the respiration of normal chicken erythrocytes (29), suggesting that its effect on survival of *P. lophurae* was a direct one.

With respect to the second question, the evidence indicates that the parasites surviving *in vitro* continue to develop, especially during the first few days. In the most favorable survival tests the parasite number maintained itself during the first 2 days and, rarely, increased slightly during the 1st day. Moreover, the percentage of very young forms devoid of pigment, and of segmenters, varied from day to day. The finding of appreciable numbers of very young forms, as well as segmenters and intermediate forms (Fig. 2), after 3 or 4 days *in vitro*, is considered to be an indication that the infection of new red cells occurred. The problem encountered by Hewitt (11) of the failure of merozoites to break out of the host cell did not arise in the present studies with *P. lophurae*. Numerous free merozoite clusters could be found. The presence of fairly numerous very young forms, as well as segmenters, was generally correlated with good survival as judged by infectivity. On the other hand, the presence of many segmenters with very few young forms was usually accompanied by relatively poor survival as judged by infectivity. It is unfortunate that *P. lophurae* exhibits such a low synchronicity (24) that more accurate data on these points are difficult to obtain. The repeated observation of more exflagellants on the 2nd, 3rd, 4th, or even 5th day of life *in vitro* than on preceding days again suggests that development of the male gametocytes occurred *in vitro* and that we are not dealing with a mere survival of gametocytes already ripe at the time of preparation of the survival tests. Indeed, the conditions of preparation were usually such that the parasite suspension was exposed to room temperature for more than 20 minutes and active exflagellation of the male gametocytes, already ripe at the time of preparation, occurred in it.

SUMMARY

The survival of *Plasmodium lophurae in vitro*, at temperatures of 39.5–42°C, is favored by a balanced salt solution having a high potassium content, by aeration but not by a very high oxygen tension, by an optimal density of parasites per cubic millimeter, by frequent renewal of the suspending medium, by concentrated red cell extract, by optimal concentrations of plasma or serum, of chick embryo extract, of glucose or glycogen, and of glutathione, and probably by yeast extract and a very low concentration of liver extract.

In the best preparations, as judged by infectivity, more than 40 per cent of the parasites were alive on the 3rd day, more than 20 per cent on the 4th day, perhaps 1 per cent on the 5th day, and about 0.05 per cent on the 6th day. Evidence was obtained that the parasites had multiplied during the 1st day of incubation.

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EXPLANATION OF PLATE 23

FIG 1 25 ml Erlenmeyer flask holding 2 ml of medium and capped with vial sealed with parafilm, as used for most of the survival tests $\times 0.6$

FIG 2 Portion of a Giemsa-stained smear from a survival test after 3 days of incubation at 41.5–42°C (Preparation 2*b* of Table VII) Note the segmenter, two young forms, and the delicately stained large trophozoite $\times 1569$

Photographed by Julian A. Carlile



(Trager Conditions affecting survival of malarial parasite)

INFLUENCE OF EXTRANEOUS PROTEIN AND VIRUS CONCENTRATION ON THE INACTIVATION OF THE RABBIT PAPILLOMA VIRUS BY X-RAYS

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Studies on γ ray irradiation of viruses have been complicated by their great resistance to the rays and as a group they are considered to be among the most radio-resistant of biologically active agents. The Shope papilloma virus (1) has been reported to be particularly resistant to γ ray irradiation, requiring a dose of 14,000,000 r to abolish the infectivity of cell free suspensions of the virus (2). We have investigated the factors responsible for the resistance of the papilloma virus to x rays¹ and it will be shown that the amount of irradiation required to inactivate the virus is not a fixed quantity but is greatly influenced by the concentration of virus in saline or buffer suspensions and by the presence of extraneous protein in the virus preparation. The findings bear upon the mechanism of x ray effects *in vitro* and will be considered in this relation.

Material and Methods

Virus was obtained from the naturally occurring papillomas of cottontail rabbits which had been plucked and preserved in 50 per cent glycerin Locke's solution in the refrigerator at about 4 C. Weighed portions of the papillomas were washed in several changes of isotonic saline, ground in a mortar to a smooth paste, and suspended in 10 or 20 volumes of 0.9 per cent saline. The crude extract was spun at about 3500 R.P.M. for 5 minutes in an angle head centrifuge, the sediment discarded and the supernatant fluid again spun at about 4500 R.P.M. for 15 to 30 minutes after which it was filtered through Berkefeld V candles. The final virus filtrate was usually highly infectious and had a clear, amber color. Purified suspensions² of the virus

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¹ Preliminary note in the *Proceedings of the Society for Experimental Biology and Medicine* 1940 45, 713.

The term 'purified virus' is used throughout this paper to mean that the virus preparation contained considerably less extraneous material than the original virus filtrate.

were prepared by centrifuging the filtrate at 30,000 R P M for 1 hour in celluloid tubes in an air driven centrifuge, after which the supernatant fluid, which contained little or no detectable virus but much extraneous protein, as indicated by nitrogen determination, was removed and discarded. The small gelatinous pellet of sediment was then resuspended in isotonic saline or 0.05 M phosphate buffer solution, pH 6.6, to the original volume and spun at about 4500 R P M for 20 minutes in the angle centrifuge. The alternate high and low speed centrifugations were repeated until the virus had been washed two to three times. Further differential centrifugations were not done because in most of the experiments not more than 24 hours elapsed from time of purification to inoculation. The final suspensions of purified virus were faintly opalescent and contained less than 1 per cent as much nitrogen as the virus filtrates.

The virus preparations were tested by pathogenicity and complement fixation tests, both already described (3, 4). Normal domestic rabbits of the gray-brown (agouti) breed obtained from local dealers were used in the *pathogenicity tests*. The inocula were rubbed into scarified skin areas on the abdomens of the test rabbits and the resulting growths recorded every 2 to 3 days from the 8th to the 24th days, and at 3 to 5 day intervals thereafter until about the 42nd day, according to a standard scale: **** = confluent papillomas, *** = semiconfluent papillomas, ** = many discrete growths, * = 5 to 15 papillomas, ± = 2, 3, or 4 papillomas, ½ = 1 papilloma, 0 = negative. (Asterisks are used in the tables for the infectivity readings to differentiate them from the plus signs used for the readings of the complement fixation tests.)

In the *complement fixation tests* various dilutions of the virus preparations were mixed with 2 units of complement (titrated immediately beforehand) and an optimal dilution of an immune serum, procured from a rabbit bearing virus induced papillomas or one that had received intraperitoneal injections of papilloma virus. The mixtures were allowed to stand at room temperature for 2 hours to allow fixation of complement and then sensitized red cells were added. Readings were made after 30 minutes in a water bath at 37°C and again after the tubes had stood overnight in a refrigerator. The latter readings are recorded in the tables as follows, in terms of fixation: +++ = complete fixation (no hemolysis), ++ = about 75 per cent fixation, + = about 50 per cent fixation, ½ = about 25 per cent fixation, ± = about 10 per cent fixation, 0 = no fixation (complete hemolysis). There is considerable evidence that the virus and the complement-fixing antigen are closely associated, if not identical (4, 5), and the complement fixation test thus provides an additional method of testing the effect of x-rays on papilloma virus preparations.

The virus suspensions were irradiated in specially made discoid flasks of pyrex glass, 1.8 cm. deep with thin top and bottom, or in small sealed glass or celluloid tubes 4 cm. long, with an internal diameter of 9 mm. Using the latter method two to four tubes could be simultaneously irradiated. They were placed half way between two x-ray tubes at a distance from each target of 10 cm. To control the temperature the containers were placed in a Petri dish or a celluloid dish containing water and the air just above was circulated with a fan. Under these conditions the temperature of the water bath never rose above 30°C. The water-cooled tubes were run at 30 ma. and at a peak voltage of about 185 kv. The x-rays were not filtered and the half value layer of radiation was 0.19 mm. of copper. The intensity was 6,200 r per minute in air as measured by a thimble chamber which had been compared with a standard

ionization chamber (6). Small samples of the virus fluid were removed from the flasks at intervals during the irradiation or when the small glass or celluloid tubes were used comparable samples of the virus fluid in different tubes were exposed to varying doses of x ray irradiation. The larger doses of irradiation used in Experiments 1 and 4 were of necessity intermittent and the virus specimens were kept in the ice box between exposures to the x rays. Control portions of the virus solutions were submitted to identical conditions but were not irradiated.

λ Ray Irradiation of a Papilloma Virus Filtrate

As a first step toward studying the effect of x rays on the papilloma virus, a Berkefeld V filtrate of highly infectious cottontail rabbit papillomas was exposed to amounts of x rays varying from 500,000 r to 11,000,000 r.

Experiment 1—A 10 per cent Berkefeld V filtrate of the glycerolated papillomas of W. R. 128 was prepared as described above. The filtrate contained 0.519 mg of nitrogen per cc. Thirty cc of the filtrate was exposed to x rays in a glass flask, under the conditions described above and 2 cc. samples of the virus fluid were removed after 1/2, 1, 2, 4, 8, and 11 million r of irradiation. A control sample of the virus filtrate was exposed to identical conditions but was not irradiated. The irradiated virus filtrate showed striking changes in the gross as the amount of x rays applied to it increased. 1,000,000 r caused a slight increase in bluish opalescence and this increased with dosage until after 8,000,000 r a heavy, flocculant precipitate had formed which settled to the bottom of the tube leaving a water-clear supernatant fluid. The control and irradiated specimens of the virus filtrate were then rubbed into scarified skin areas of three normal domestic rabbits.

The results of the experiment are shown in Table I. The papillomas elicited by the specimen of the virus filtrate that received 500,000 r of irradiation were slightly smaller and fewer in number than the growths produced by the control virus filtrate a fact evident in the table on the 14th and 24th days after virus inoculation. 1,000,000 r of irradiation caused a marked decrease in the number of papillomas elicited by the virus filtrate, and the incubation period of the growths was considerably longer than that of the controls. Only a few growths, and these with a long incubation period, were obtained with the virus specimen that received 2,000,000 r and no papillomas were elicited by the filtrate after exposure to 4,000,000 r or more of x ray irradiation. These findings were confirmed by inoculating the virus specimens into another group of four domestic rabbits. All of the growths induced proved to be benign papillomas of the ordinary sort.

Effect of λ-Rays on the Complement Binding Capacity of a Papilloma Virus Filtrate

The available evidence indicates that the capacity of a papilloma virus suspension to fix complement in mixture with specific immune serum is a property of the virus *per se* or of an integral part of it (4, 5, 7). A comparison of the effect

of x-rays on the infectivity and the complement-fixing capacity of a papilloma virus filtrate has for this reason considerable interest

Experiment 2—The control and irradiated specimens of the virus filtrate W R 1-28, described in Experiment 1, were tested in various dilutions immediately after irradiation for capacity to fix 2 units of complement in mixture with an immune serum (D R 10) obtained from a rabbit which had borne virus-induced papillomas and which in addition had received two intraperitoneal injections of a 5 per cent virus filtrate to call forth serum antibody in high titer

TABLE I
Effect of Irradiation on a Papilloma Virus Filtrate

Virus filtrate W R 1 28 X-ray irradiation	Pathogenicity tests								
	14th day			24th day			42nd day		
	a	b	c	a	b	c	a	b	c
<i>roentgens</i>									
Control	****	**	***	****	****	****	****	****	****
500,000	**	*	**	****	***	***	****	****	****
1,000,000	0	0	±	**	*	**	****	***	***
2,000,000	0	0	0	±	±	0	±	*	±
4,000,000	0	0	0	0	0	0	0	0	0
8,000,000	0	0	0	0	0	0	0	0	0
11,000,000	0	0	0	0	0	0	0	0	0

a, b, c = test rabbits

**** = confluent papillomas

*** = semiconfluent papillomas

** = many discrete growths

* = 5 to 15 papillomas

± = 2, 3, or 4 papillomas

± = 1 papilloma

0 = negative

Table II shows the results of the experiment. It will be seen that 500,000 r of x-rays had no detectable effect on the complement-binding capacity of the virus filtrate. One to 4 million r gradually reduced the titer, but 8,000,000 r was necessary to abolish completely the capacity of the filtrate to react in the test. When the findings of the complement fixation test are compared with the infectivity tests of the same virus filtrate (Table I), it is apparent that the complement-binding antigen is more resistant to x-rays than the infectious virus. 4,000,000 r completely abolished the capacity of the filtrate to elicit papillomas, but it still reacted in the complement fixation test in a dilution of 1/160.

Effect of X-Rays on Purified Papilloma Virus Suspensions

The papilloma virus has been shown to be a high molecular weight protein that can be readily sedimented from extracts of infectious cottontail rabbit

papillomas by high speed centrifugation (8). Since the virus protein constitutes only a small fraction of the material present in crude papilloma extracts, it seemed worth while to determine whether the extraneous material had any influence on the effect of x rays upon the virus proper.

Experiment 3—A portion of the virus filtrate (W. R. 128) used in the preceding experiments was spun at 30,000 R.P.M. for 1 hour in the air driven centrifuge and the pellet of sediment was resuspended in the original volume of 0.9 per cent saline. The suspension was then spun at about 4500 R.P.M. for 20 minutes in the angle head centrifuge; the supernatant fluid was removed and the fluid again spun at 30,000 R.P.M. for 1 hour. The small pellet of sediment was resuspended in 0.9 per cent

TABLE II
Complement Fixation Tests with a Papilloma Virus Filtrate after Irradiation

X ray irradiat on of virus filtrate W. R. 128†	Complement fixation tests‡						
	Dilutions of virus filtrate						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
<i>Results</i>							
Control	++++	++++	++++	++++	++++	++++±	++
500 000	++++	++++	++++	++++	++++	++++±	++
1 000 000	++++	++++	++++	++++	++++	+±	0
2 000 000	++++	++++	++++	++++	++++±	±	0
4 000 000	++++	++++	++++	++++	++++±	0	0
8 000 000	ac	0	0	0	0	0	0
† 10 per cent Berkefeld V filtrate	++++ = complete fixation (no hemolysis)						
‡ Complement 2 units in all tubes	+++ = about 75 per cent fixation						
Immune serum D. R. 10:148	++ = 50						
ac = anticomplementary	+ = 25						
	± = 10						
	0 = no fixation (complete hemolysis)						

saline and finally spun at 4500 R.P.M. for 20 minutes and the sediment discarded. The suspension was faintly opalescent. As additional material a freshly prepared virus filtrate (W. R. 1-70) was similarly purified. Thirty cc. of the purified virus suspensions were then irradiated, as described in Experiment 1, with removal of small samples of the virus fluid after exposures to from 100,000 r to 4,000,000 r of x ray irradiation. The irradiated virus suspensions showed little change in the gross from the control non irradiated virus suspension except for a fine granular precipitate in the samples receiving the larger doses of x ray. The control and irradiated specimens were then rubbed into scarified skin areas on three normal domestic rabbits in the usual way. In addition the specimens were tested for capacity to fix 2 units of complement in mixture with a 1:48 dilution of the immune serum D. R. 10.

The results are summarized in Table III. The control specimens were highly infectious, eliciting many discrete or semiconfluent papillomas by the 14th day after inoculation and they became large confluent papillomatous

TABLE III
Effect of Irradiation on Suspensions of Purified Papilloma Virus

Purified virus No	X ray irradiation	Pathogenicity tests									Complement fixation tests†				
		14th day			21st day			42nd day			Dilutions of virus				
		a	b	c	a	b	c	a	b	c	1 10	1 20	1 40	1 80	1 160
W R 1 23	Control	**	**	***	***	***	***	***	***	***	+++	+++	+++	+++	0
	100,000	0	0	0	0	0	*	*	*	*	+++	+++	+++	0	0
	200,000	0	0	0	0	0	0	*	*	*	+++	+++	0	0	0
	400,000	0	0	0	0	0	0	0	*	*	+++	+++	0	0	0
	800,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W R 1 70	Control	***	***	***	***	***	***	***	***	***	+++	+++	+++	+++	+++
	250,000	0	0	0	*	*	*	*	*	*	+++	+++	+++	+++	+++
	500,000	0	0	0	0	0	0	0	*	*	+++	+++	+++	+	0
	750,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1,000,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0

† 10 per cent virus filtrates were spun at 30,000 r.p.m. for 1 hour, the pellet of sediment resuspended in saline to the original volume, and the suspension spun at 4100 r.p.m. for 20 minutes to remove aggregated material. The high and low speed centrifugations were then repeated.

§ Complement, 2 units in all tubes. Immune serum, D R 10, 1 48.

*, b, c = test rabbits

masses by the 42nd day. Virus suspension W. R. 128 was completely innocuous after receiving 800,000 r of irradiation, and 100,000 r largely inactivated it. The virus specimen of W. R. 170 that had been submitted to 750,000 r or more of irradiation elicited no papillomas in any of the test rabbits. 250,000 r caused a striking reduction in the capacity of the virus to elicit papillomas, and the virus specimen that received 500,000 r caused only a few papillomas in two of the test rabbits and this after a long incubation period. The complement binding capacity of the virus preparations was abolished by the same dose of X rays required to destroy the infectivity of the virus fluids. It should be noted, however, that in both preparations the capacity to fix complement was inactivated at a slower rate than the pathogenicity of the virus. When this finding is considered together with the fact that the infectivity tests for virus have a lower threshold than do complement fixation tests (4), it is evident that the complement binding capacity of purified suspensions of virus is more resistant than the infectivity of the virus. In this respect the findings are like those with the virus filtrate described in Experiment 1, though not as striking.

The experiment (Table III) showed that suspensions of papilloma virus purified by repeated differential centrifugation were inactivated by amounts of x ray far less than had been required to render papilloma virus filtrates non-infectious. One of the purified preparations came from a filtrate (W. R. 128) several times tested in the latter respect (Experiments 1 and 2). 100,000 r inactivated most of the infectious virus of the purified preparation of W. R. 128 and 800,000 r completely abolished its capacity to elicit papillomas or to react in the complement fixation test, whereas 1 to 8 million r was required to produce the same effects on the original filtrate. The findings indicate that the amount of x ray irradiation required to inactivate suspensions of the papilloma virus is greatly influenced by the extraneous material present in the virus preparation.

To obtain more data, two other virus filtrates differing widely in pathogenicity were tested in comparison with fractions of the same filtrates after purification. Also the non-infectious supernatant fluids obtained after centrifugation of the filtrates at 30,000 R.P.M. for 1 hour were added to some of the purified virus suspensions before irradiation to make certain that the extraneous material in the filtrates made necessary the large amount of x rays which was required to inactivate the virus filtrates.

Experiment 4—10 per cent virus filtrates of the highly infectious papillomas of W. R. 171 and W. R. 50 respectively were prepared in the usual way. Previous tests had shown that the material of W. R. 50 yielded but a small amount of virus. A portion of each filtrate was spun in the air driven centrifuge at 30,000 R.P.M. for 1 hour and the supernatant fluids were carefully removed. One half of the sediment of each filtrate was then resuspended to the original volume in 0.9 per cent saline.

The remaining portion of the virus sediment of W R 1-71 was resuspended in the supernatant fluid from the filtrate of W R 50 obtained after the high speed centrifugation while the sediment of W R 50 was resuspended in the supernatant fluid of W R 1-71. All of the resuspended suspensions were then spun at about 4500 R P M for 1 hour. Nitrogen determinations showed that virus filtrate W R 1-71 contained 0.557 mg per cc, the purified virus fraction 0.028 mg per cc, and the fraction resuspended in the supernatant fluid of the W R 50 filtrate 0.34 mg nitrogen per cc. Virus filtrate W R 50 contained 0.335 mg per cc, the purified virus fraction 0.021 mg per cc, and the purified fraction suspended in the supernatant fluid of W R 1-71 0.566 mg per cc. The virus filtrates and the purified saline suspensions of each, as well as the supernatant fluids removed from the filtrates after centrifugation of the latter at 30,000 R P M for 1 hour were rubbed into scarified areas of three domestic rabbits. In addition each preparation was tested for capacity to fix two units of complement in mixture with immune serum D R 2 diluted 1:16 in saline.

The results are summarized in Table IV. It will be seen that virus filtrate W R 1-71 contained considerably more virus and complement-binding antigen than virus filtrate W R 50. The purified preparations of the filtrates were only slightly less infectious and reacted in slightly lower titer in the complement fixation test than the filtrates. The supernatant fluids, on the other hand, contained practically no virus (the supernatant fluid of the W R 1-71 filtrate elicited a solitary growth in only one of the test rabbits) and had no capacity to react in the complement fixation test.

The virus materials were irradiated in small sealed glass tubes, each containing 1 cc of one or another of the various fluids. Each tube was exposed to a different amount of x-rays, from 100,000 r to 2,000,000 r, and the specimens were rubbed into scarified skin areas of three domestic rabbits.

Table V shows the results of the irradiation. The highly infectious virus filtrate W R 1-71 elicited a few discrete papillomas in two of three test rabbits after exposure to 2,000,000 r of x-ray irradiation. The purified suspension of the filtrate, on the other hand, was almost completely inactivated by 500,000 r. The purified preparation of the virus suspended in the supernatant fluid of the W R 50 filtrate was as resistant as the original filtrate to the effects of the x-rays. It should be noted that the same amount of virus was present in the purified virus preparation suspended in saline as in the preparation suspended in the supernatant fluid of the filtrates, a fact confirmed by the results of infectivity tests (not given in detail in the table). Hence the difference in sensitivity of the two preparations to x-rays can only have been due to the presence of the extraneous material in the latter. Similar results were obtained with the W R 50 virus preparations. 2,000,000 r completely inactivated the filtrate, and no papillomas were elicited by the purified fraction suspended in saline after 500,000 r of x-ray irradiation. The purified fraction suspended

TABLE IV
Infectivity and Complement Fixation Tests with Fractions of *Virus Fulvales*

Virus No	Virus preparation	Pathogenicity tests									Complement fixation tests				
		12th day			21st day			35th day			Dilutions				
		a	b	c	a	b	c	a	b	c	1 10	1 20	1 40	1 80	1 160
W R. 1 71	Whole filtrate	***	***	*	***	***	***	***	***	***	+++	+++	+++	+++	++
	(a) Resuspended sediment after centrifugation at 30 000 x g M for 1 hr	*	***	*	***	***	***	***	***	***	+++	+++	+++	+++	+
	(b) Supernatant of (a)	0	0	0	0	0	0	0	±	0	0	0	0	0	0
W R. 50	Whole filtrate	0	0	0	**	***	**	***	***	***	+++	+++	0	0	0
	(c) Resuspended sediment after centrifugation at 30 000 x g M for 1 hr	0	0	0	**	**	**	***	***	***	+++	+	0	0	0
	(d) Supernatant of (c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

^{||} Complement 2 units in all tubes Immune serum D R. 2 1 16

in the supernatant fluid of the W R 1-71 filtrate, though containing more nitrogen than any of the other preparations, was also completely inactivated

TABLE V
Influence of Extraneous Material on X-Ray Inactivation of the Virus

Virus No	Virus preparation	Nitro gen	Test rab bits	Pathogenicity tests				
				X ray irradiation				
				Control	100 000 r	500 000 r	1 000 000 r	2 000 000 r
W R 1-71	(1) Filtrate	0 557	a	****	****	****	***	±
			b	****	****	****	***	±
			c	***	***	***	**	0
	(2) Purified fraction of (1) suspended in saline†	0 028	d	****	****	±	0	0
			e	****	****	0	0	0
			f	****	****	±	0	0
	(3) Purified fraction of (1) suspended in non infectious super- natant fluid of W R 50 filtrate§	0 340	a	****	****	****	****	*
			b	****	****	****	****	*
			c	***	***	**	**	±
W R 50	(4) Filtrate	0 335	a	****	****	***	**	0
			b	****	****	***	**	0
			c	***	**	**	*	0
	(5) Purified fraction of (4) suspended in saline†	0 021	d	***	**	0	0	0
			e	***	***	0	0	0
			f	***	***	0	0	0
	(6) Purified fraction of (4) suspended in non infectious super- natant fluid of W R 1-71 filtrate§	0 566	a	****	***	***	0	0
			b	****	***	***	±	0
			c	***	**	**	±	0

† Portion of the filtrate spun at 30,000 R P M for 1 hour, the supernatant fluid removed, and the pellet of sediment suspended in saline to the original volume

§ Same as above, except that the pellet of sediment was suspended in the supernatant fluid of a filtrate after centrifugation at 30,000 R P M for 1 hour

| Growths on the 42nd day after virus inoculation

by 2,000,000 r of irradiation. The W R 50 virus preparations contained considerably less virus than the W R 1-71 preparations (Table IV), and it seems likely that this will account for the fact that more irradiation was required to inactivate the latter preparation.

In another experiment the effect of x rays on the complement fixing antigen of purified papilloma virus preparations of widely different capacities was tested (Table VI). 700 000 r of x ray irradiation was necessary to abolish the complement binding capacity of the W R 295 virus preparation which contained much of the antigen, whereas the W R D virus preparation that contained considerably less was completely inactivated after exposure to 300 000 r. The findings (Tables V and VI) show that virus suspensions containing much virus required more irradiation to abolish the infectivity and the capacity to fix complement than comparable preparations containing less virus.

TABLE VI

Effect of Irradiation on the Complement Fixing Capacity of Purified Virus

X ray irradiation	Complement fixation test									
	Purified virus W R. 295						Purified virus W R. D			
	Dilutions						Dilutions			
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80
<i>results</i>										
Controls	++	++++±	+++++	+++++	+++++	±	+++++	+++++	±	0
100 000	+	+++++	+++++	+++++	+++	0	+++	±	0	0
300 000	+++++	+++++	+++++	+++++	±	0	0	0	0	0
500 000	++	±	0	0	0	0	0	0	0	0
700 000	0	0	0	0	0	0	0	0	0	0

* Complement 2 units in all tubes

Immune serum F2 1:16

** Two differential centrifugations

Influence of Normal Rabbit Serum on the Effects of Irradiation

Will normal rabbit serum—which is wholly innocuous for the virus—influence the effect of x rays on purified suspensions of the papilloma virus?

Experiment 5—A 10 per cent virus filtrate (W R. 1-30) was purified by two differential centrifugations, as previously described. The material was resuspended to the original bulk. An equal volume of fresh normal rabbit serum diluted 1:10 with isotonic saline was added to a portion of the purified virus preparation and to another portion an equal volume of 0.9 per cent saline was added. The suspension in rabbit serum contained 0.887 mg. nitrogen per cc. while that in saline contained only 0.0078 mg. of nitrogen per cc. The virus preparations were then exposed to x rays in amount from 100 000 r to 800 000 r in small sealed glass tubes. Comparable preparations of the virus in serum and in saline were irradiated together. Inoculation was done into three normal rabbits and the complement fixation test was also carried out.

The results are summarized in Table VII. The virus suspended in saline was largely inactivated by 100 000 r of x ray irradiation. After exposure to

TABLE VII
Effect of virus on the development of virus in the cytoplasm of cells

Inoculum	Inoculum	Days of incubation						Days of incubation					
		1st day			2nd day			3rd day			4th day		
No. of cells	No. of cells	a	b	c	a	b	c	a	b	c	a	b	c
		0	0	0	0	0	0	0	0	0	0	0	0
0.0 per cent saline	Control	0	0	0	0	0	0	0	0	0	0	0	0
	100 (0)	0	0	0	0	0	0	0	0	0	0	0	0
	50 (0)	0	0	0	0	0	0	0	0	0	0	0	0
	25 (0)	0	0	0	0	0	0	0	0	0	0	0	0
10 per cent normal rabbit serum	Control	0	0	0	0	0	0	0	0	0	0	0	0
	100 (0)	0	0	0	0	0	0	0	0	0	0	0	0
	50 (0)	0	0	0	0	0	0	0	0	0	0	0	0
	25 (0)	0	0	0	0	0	0	0	0	0	0	0	0

† Two differential centrifugations

‡ Complement, 2 units in all tubes

a, b, c = test rabbits

Immune serum, D R 10, 148

400,000 r it elicited no papillomas and failed to react in the complement fixation test. The same virus suspended in 10 per cent normal rabbit serum, on the other hand, was still highly infectious after exposure to 800,000 r though partial inactivation had occurred, and it still reacted in the complement fixation test in a dilution of 1:40, the control specimen reacted in dilution of 1:80. Manifestly normal rabbit serum protects the papilloma virus from doses of x-ray that are sufficient to destroy the virus in saline suspension.

Influence of Crystalline Egg Albumin on the Effects of X-Ray Irradiation

These results made it seem likely that the extraneous protein in the virus filtrates and in rabbit serum protected virus from the x-rays. In a further test a purified virus preparation which had been suspended in a phosphate buffer solution and in a solution of crystalline egg albumin, respectively, were exposed simultaneously to the rays.

Experiment 6—A 5 per cent virus filtrate of the papillomas of W. R. 295 was purified by three differential centrifugations. The pellets of virus sediment obtained by the high speed centrifugations were resuspended in the original volume of 0.05 M phosphate buffer solution, pH 6.6. The final virus preparation had 0.0014 mg of nitrogen per cc. A portion of the virus suspension was then diluted tenfold with the buffer solution and another portion similarly diluted with a solution of crystalline egg albumin. The egg albumin was kindly supplied by Dr. A. E. Mirsky. The original solution of it had been crystallized three times and contained 75.3 mg protein per cc. but it was diluted with the buffer solution before mixing with the virus suspension so that the final virus-egg albumin suspension had a nitrogen content of 0.712 mg per cc., which is comparable to the nitrogen content of papilloma virus filtrates. The two virus preparations were simultaneously exposed to 100,000 r of x-rays in small glass tubes. The control and irradiated virus fluids were then rubbed into scarified skin areas of six domestic rabbits.

The results of the experiment are shown in Table VIII. There was no difference in the infectivity of the control suspensions of virus in buffer and in buffer plus egg albumin, both eliciting about the same number of papillomas with comparable incubation periods in the test rabbits. There was a striking difference, however, in the effect of x-rays on the two virus preparations. 100,000 r completely inactivated the virus suspended in the buffer solution whereas the same amount of x-rays had only a slight effect on the infectivity of the virus suspended in the solution of egg albumin.

Relation of Virus Concentration to Percentage Inactivation by X-Rays

Because of the influence of extraneous protein on the radiation effect, it seemed of interest to compare the percentage inactivation of a concentrated and a buffer diluted virus solution by the same amount of x-rays.

TABLE VIII
Activity inactivation of Virus Suspended in Buffer with or without 1% egg white

Inactivation Buffer	Inoculum	No. of inoculations	Laboratory											
			1st day			4th day			10th day			14th day		
			a	b	c	d	e	f	a	b	c	d	e	f
Buffer with 1% egg white	0.05 M	Control	0	0	0	0	0	0	0	0	0	0	0	0
	0.05 M	Control	0	0	0	0	0	0	0	0	0	0	0	0
Buffer with 1% egg white	0.05 M	Control	0	0	0	0	0	0	0	0	0	0	0	0
	0.05 M	Control	0	0	0	0	0	0	0	0	0	0	0	0

0.05 M phosphate buffer, pH 6.6

1% egg white crystallized 3 times

a, b, c, d, e, f = test rabbits.

Experiment 7—A 5 per cent Berkefeld V filtrate of the papillomas of W R E (pooled, naturally occurring growths from seven Kansas cottontails) was prepared in the usual way. The filtrate, which contained 0.287 mg of nitrogen per cc, was purified by two differential centrifugations, with resuspension of the pellets of virus sediment in 0.05 M phosphate buffer solution, pH 6.6. To obtain a concentrated suspension of virus, the final volume of buffer solution used was one fourth that of the original volume of virus filtrate. The final purified suspension had 0.00896 mg of nitrogen per cc. It was exposed together with a portion diluted with 20 volumes of buffer to 50,000 r of x-ray irradiation. Irradiation was done in small glass tubes of 1 cc volume. As controls, portions of the concentrated and diluted virus fluids were not irradiated but were submitted to identical conditions in other respects. Six normal domestic rabbits were inoculated with the materials. Each was rubbed into two skin areas of every rabbit, so that a total of twelve areas received each virus fluid. The animals were carefully examined at 2-day intervals for the time of appearance of the papillomas, since the incubation period (the time from virus inoculation to appearance of the growths) is largely determined by the amount of virus present in the inoculum (9).

The results of the experiment are summarized in Table IX. The concentrated control virus fluid was highly infectious, eliciting papillomas in the test rabbits after a mean incubation period of 10.2 days, the growths rapidly forming large confluent papillomatous masses. The virus specimen that had received 50,000 r of x-ray irradiation was only slightly less infectious than the control virus fluid, as evidenced by a mean incubation period of 11.0 days. Almost as many papillomas were elicited as by the control virus fluid. The control virus fluid, that had been diluted twentyfold, elicited papillomas with a mean incubation period of 14.2 days. The diluted virus suspension that had received 50,000 r of x-ray irradiation, on the other hand, was largely inactivated, eliciting only a few discrete papillomas in nine of the twelve inoculated areas, and the average incubation period of the growths that appeared was 21.0 days.

The wide difference in the percentage inactivation of the virus was attested also by difference in the incubation period of the papillomas.

The fact is well known (1, 9) that the incubation period varies with the amount of papilloma virus inoculated. For the purposes of the present work three experiments were done with a virus filtrate and two purified suspensions respectively to learn the effect of a twentyfold dilution of a highly pathogenic virus preparation on the incubation period. The period was found to be lengthened by 4.0 to 4.5 days. Using this value and the form of the relationship found by Bryan and Beard (9) it is possible to calculate roughly the percent of virus remaining infectious in irradiated virus fluids. It would appear that in Experiment 7 56 per cent of the concentrated virus suspension remained infectious after 50,000 r of irradiation but only 0.7 per cent when the preparation had been diluted twenty times before irradiation.

To enlarge the findings a more comprehensive experiment was done, using another purified virus preparation and amounts of x-rays ranging from 3,000 r to 50,000 r.

TABLE IX
Infectiousness of virus after incubation at 37°C

Incubation time (hr)	Incubation temperature (°C)										Total number of rabbits	Percentage of rabbits infected
	a	b	c	d	e	f	g	h	i	j		
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	0	0	0
54	0	0	0	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0
58	0	0	0	0	0	0	0	0	0	0	0	0
59	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0
61	0	0	0	0	0	0	0	0	0	0	0	0
62	0	0	0	0	0	0	0	0	0	0	0	0
63	0	0	0	0	0	0	0	0	0	0	0	0
64	0	0	0	0	0	0	0	0	0	0	0	0
65	0	0	0	0	0	0	0	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0	0	0	0
67	0	0	0	0	0	0	0	0	0	0	0	0
68	0	0	0	0	0	0	0	0	0	0	0	0
69	0	0	0	0	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	0	0	0	0	0	0
71	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0	0	0	0
73	0	0	0	0	0	0	0	0	0	0	0	0
74	0	0	0	0	0	0	0	0	0	0	0	0
75	0	0	0	0	0	0	0	0	0	0	0	0
76	0	0	0	0	0	0	0	0	0	0	0	0
77	0	0	0	0	0	0	0	0	0	0	0	0
78	0	0	0	0	0	0	0	0	0	0	0	0
79	0	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	0	0
81	0	0	0	0	0	0	0	0	0	0	0	0
82	0	0	0	0	0	0	0	0	0	0	0	0
83	0	0	0	0	0	0	0	0	0	0	0	0
84	0	0	0	0	0	0	0	0	0	0	0	0
85	0	0	0	0	0	0	0	0	0	0	0	0
86	0	0	0	0	0	0	0	0	0	0	0	0
87	0	0	0	0	0	0	0	0	0	0	0	0
88	0	0	0	0	0	0	0	0	0	0	0	0
89	0	0	0	0	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0	0	0	0	0
91	0	0	0	0	0	0	0	0	0	0	0	0
92	0	0	0	0	0	0	0	0	0	0	0	0
93	0	0	0	0	0	0	0	0	0	0	0	0
94	0	0	0	0	0	0	0	0	0	0	0	0
95	0	0	0	0	0	0	0	0	0	0	0	0
96	0	0	0	0	0	0	0	0	0	0	0	0
97	0	0	0	0	0	0	0	0	0	0	0	0
98	0	0	0	0	0	0	0	0	0	0	0	0
99	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0

Two differential centrifugations, virus suspended in 0.05 M phosphate buffer solution, pH 6.6

§ Cultured from incubation period of papillomas (see text)

a, b, c, d, e, f = test rabbits

Experiment 8—A 5 per cent virus filtrate (W R 2 95) was purified by three differential centrifugations, with resuspension of the pellets of sediment in 0.05 M phosphate buffer solution pH 6.6. The final pellets of sediment were resuspended in one fourth of the original volume of the buffer solution in order to obtain a potent virus preparation. The virus fluid was faintly opalescent and had 0.028 mg of nitrogen per cc. The virus suspension in the original concentration and a portion diluted twentyfold were then simultaneously exposed together to 50,000 r of x ray irradiation in 1 cc volumes in sealed glass tubes, as previously described. The twentyfold dilution of the virus fluid and a portion diluted to 1/1000 were similarly irradiated with 10,000 r while another portion of the latter dilution of virus received only 3,000 r. The specimens were inoculated into six normal domestic rabbits, each into two areas of each rabbit, save for the specimen exposed to 3,000 r which was inoculated into only one area on each rabbit. As in the preceding experiment, the animals were carefully examined at 2 day intervals for papillomas.

The results are summarized in Table V. The concentrated virus suspension that received 50,000 r of x ray irradiation elicited only slightly fewer papillomas than the control non irradiated virus fluid yet the same dose of x ray almost completely inactivated the virus suspension diluted twenty times, the control virus fluid remaining highly infectious. The difference in incubation period of the control and irradiated concentrated virus fluids was 1.3 days, indicating that 40 per cent of the virus was still infectious after irradiation. The incubation period of the virus fluid that had been diluted twenty times was increased from 14.3 to 24.3 days from which one may infer that only 0.08 per cent of the virus was still active. 10,000 r had only a slight effect on the number of papillomas elicited by the virus suspension diluted twenty times but it lengthened the incubation period from 14.3 to 15.7 days (about 38 per cent of the virus remaining infectious), while the same dose of x ray almost completely inactivated the virus diluted 1,000 times and increased the incubation period of the papillomas that appeared by 4.8 days (3.1 per cent of the virus remaining pathogenic). 3,000 r of x ray irradiation had a pronounced effect upon the virus in the suspension diluted 1,000 times. The irradiated virus fluid elicited only a few growths in four of the six test rabbits while the control virus fluid caused many discrete papillomas in all, and the difference in the incubation period of the papillomas elicited by the control and irradiated virus specimens was 1.7 days, (28 per cent of the virus remaining infectious).

Complement fixation tests with the above virus materials showed that 50,000 r of irradiation had no significant effect on the complement binding antigen of the concentrated virus suspension and the same amount of x ray inactivated less than 50 per cent of the antigen in the virus suspension diluted twenty times.

The findings of Experiments 7 and 8 show that the percentage inactivation of papilloma virus in purified suspensions increases as the concentration of virus is decreased by dilution (Tables IX and V). It is remarkable that the low dose of 3,000 r inactivated over 70 per cent of the virus in a suspension diluted 1,000 times, yet 50,000 r was necessary to produce roughly the same percentage inactivation of the purified virus in concentrated suspension.

TABLE V
 Latency in the cells of the virus

Incubation period (days)	Incubation period (days)												Total number of cells	Percentage of cells with virus
	a	b	c	d	e	f	g	h	i	j	k	l		
11	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
12	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
13	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
14	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
15	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
16	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
17	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
18	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
19	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
20	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
21	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
22	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
23	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
24	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
25	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
26	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
27	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
28	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
29	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
30	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
31	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

† Three differential centrifugations, virus suspended in 0.05 M phosphate buffer, pH 6.6

‡ Calculated from incubation period of papillomas (see text)

a, b, c, d, e, f = test rabbits

In a further experiment the percentage inactivation of a virus filtrate following irradiation also increased when the filtrate was diluted with saline. The percentage inactivation did not increase, however, when the same filtrate was diluted with the non infectious supernatant fluid of a portion of the filtrate.

λ Ray Irradiation of Serum Antiviral Antibody

It seemed likely from the preceding experiments and the results on irradiation of enzymes (17-19) that the factors of concentration and content of extraneous protein would influence the inactivation by x rays of other proteins having different biological functions. To determine the effect of x rays on serum antibodies was tested in the next experiment.

The papilloma virus elicits a specific serum antibody that is capable of neutralizing the virus or of fixing complement in mixture with it (4). Studies on the immunological reactions of the virus and its specific antibody have shown that the virus, or an integral part of it, is responsible for both reactions (4, 5, 7), and that the complement fixation test affords a reliable method of demonstrating the antibody specifically directed against the papilloma virus. Furthermore the antiviral antibody can be partially purified by precipitating the globulin fraction from whole rabbit serum with ammonium sulfate (10). Consequently the effect of x rays on the complement binding antibody in whole immune rabbit serum and in the fraction of serum precipitated with half saturated ammonium sulfate was compared in the next experiment.

Experiment 9—Immune serum was obtained from a domestic rabbit (D R 14-71) which had received two intraperitoneal injections of 10 cc. of a 10 per cent papilloma virus filtrate (W R 295). The serum was diluted 1:4 with isotonic saline. A portion was mixed with an equal volume of saturated ammonium sulfate and after standing at room temperature for 30 minutes the precipitate was sedimented by centrifugation at about 4500 R.P.M. for 10 minutes. It was then resuspended in the original volume of saline and dialyzed against isotonic saline overnight in the refrigerator. Nitrogen determinations showed that the original serum (diluted 1:4) had 2.66 mg. of nitrogen per cc. and the globulin fraction 0.726 mg. Both were exposed together to 500,000 r of x rays in celluloid tubes. The control and irradiated serum specimens were then tested for capacity to fix 2 units of complement in mixture with a papilloma virus extract (W R D) diluted 1:160 as the antigen.

The results of the experiment are shown in Table XI. 500,000 r of x ray irradiation had only a slight effect on the complement binding antibody of the whole serum, reducing the titer from complete fixation in dilution of 1:128 to partial fixation at this dilution. The globulin fraction of the serum, however, which contained as high a titer of antibody as the original whole serum, failed to react in the complement fixation test after irradiation. Evidently the antiviral antibody in whole serum is considerably more resistant to x rays than

ACTIVATION OF RABBIT PAPILLOMA VIRUS

the serum was precipitated in the globulin fraction of the same serum. In a subsequent experiment, the immune serum and its globulin fraction was exposed to 250,000 r of x-ray irradiation. In addition a portion of the serum was exposed to gamma-ray irradiation.

Experiment 1. An immune serum (D. R. 11-73), from a domestic rabbit which had been immunized by an intradermal injection of 10 cc. of a 10 per cent papilloma virus extract was diluted 1:4 in dilute phosphate buffer solution pH 7.2 and a portion of the mixture was exposed to 100,000 r of x-ray irradiation. After standing for 24 hours at room temperature the mixture was spun at about 4500 R.P.M. for 20

TABLE XI
Effect of X-rays on the Antititer of Antisera

			Serum antibody titer as determined by complement fixation tests:						
			Dilutions of serum						
Antiserum	Conc.	Exposure	1:4	1:8	1:16	1:32	1:64	1:128	1:256
W. R. 128	0.72%	Control	++++	++++	++++	++++	++++	++++	0
		50,000 r	++++	++++	++++	++++	++++	+	0
D. R. 11-73	0.72%	Control	++++	++++	++++	++++	++++	++++	0
		50,000 r	0	0	0	0	0	0	0

* 1 cc. of the serum was diluted with 3 cc. of isotonic saline and dialyzed against isotonic saline.

* Control serum was tested in all tubes.

* Serum W. R. 128 was extract 1:160.

* None of the sera was an agglutinating when tested in double amounts.

minutes and the sediment resuspended in the original volume of isotonic saline. The sediment was again precipitated with an equal volume of saturated ammonium sulfate. The sediment after centrifugation was resuspended in a small volume of isotonic saline and dialyzed overnight against saline in the refrigerator. The volume was adjusted to three fourths of the original volume with the phosphate buffer solution pH 7.2 in order to compensate for the loss of antibody incident to the dialysis. The serum was then tested and it was found that the whole serum (1:4) contained 250,000 r of x-ray exposure and the globulin fraction 0.739 mg per cc. The serum was then diluted 1:42 and 1:48 with buffer solution and the globulin fraction of the serum was exposed to 250,000 r of x-ray irradiation in sealed celluloid tubes. The serum and globulin preparations were not irradiated but were subjected to gamma-ray irradiation. The specimens were then tested for capacity to precipitate papilloma virus extract (W. R. 128).

Experiment II. In a subsequent experiment the antibody titer of the immune serum (D. R. 11-73) was only slightly reduced by 250,000 r of

TABLE VII
Influence of Dilution and of Extraneous Material on the Inactivation of Serum Antibody by X Rays

Immune serum D R 1473	Nitrogen	X ray irradiation	Titer of serum antibody as determined by complement fixation tests ¹													
			Serum diluted 1:4 before irradiation							Serum diluted 1:48 before irradiation						
			Dilutions of serum							Dilutions of serum						
	mg per cc		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:48	1:96	1:192	1:384	1:768	1:1536	1:3072
Whole serum	2.633	Control 250,000+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Globulin fraction ²	0.739	Control 250,000+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

* Whole serum diluted 1:4 with 0.05 M phosphate buffer pH 7.2

† Serum (1:4) precipitated with 1/2 saturated ammonium sulfate dialyzed against isotonic saline and brought to original volume with buffer pH 7.2

‡ Complement 2 units in all tubes
Antigen W R 128 virus extract, 1:120

The concentration of 1:10, however, was almost completely inactivated by gamma-rays. Comparison indicates that irradiation inactivated about 25 per cent of the antibody in the concentrated serum, but about 75 per cent of the serum was diluted twelve times. Similar findings were obtained in another experiment when a globulin fraction of an immune serum was irradiated in concentrated and diluted form.

Results of Experiments 9 and 10 prove that the effect of x-rays on the serum antibody is greatly influenced by other substances in the serum. Not only is the radiation more effective when the serum albumin is removed, but the percentage inactivation of serum antibody by x-rays increases as serum is diluted. When the findings are compared with the results with the papilloma virus it is plain that the same factors influence the inactivation by x-rays of both large and small molecular weight proteins.

DISCUSSION

The experiments here reported provide evidence that the papilloma virus is more resistant to x-rays as had been thought (2). The presence of extraneous material is largely responsible for the ineffectiveness of the rays. In our experiment, 2 to 4 million r are required to inactivate Berkeley filtrates of the virus whereas 50,000 to 400,000 r sufficed when it had been previously subjected to differential centrifugations. Dilution of papilloma virus with physiological saline or buffer solutions also reduced the dose of x-rays required. When the virus was greatly diluted, as little as 3,000 r inactivated a part of it. This dose of x-ray is even less than that required to induce x-ray induced papillomas of domestic rabbits to regress *in vivo* (11, 12). It seems likely, however, that the effect of x-rays in causing the papilloma to regress is exerted on the cell, though not because of the resistance of the papilloma virus, as previously postulated, but because of the protection afforded the latter by cell materials. Purified and diluted suspensions of virus are more resistant to x-rays than are some bacteria, protozoa, and yeasts.

The findings here make questions as concerns the mechanism of the action of x-rays on viruses and probably on other large molecular weight proteins under certain conditions. Since penetrating radiation was used only a small fraction of the total dose absorbed by the virus suspensions.² It follows from these results that some effect of the type observed with the non-penetrating

² The dose absorbed by the concentrated and a concentrated suspension of purified virus was 100,000 r. The dose absorbed by a diluted suspension of the virus was 10,000 r. The dose absorbed by the 50,000 r of radiation passed through both suspensions was 10,000 r. The results of incubation of material irradiated in the concentrated suspension and in the diluted suspension were similar.

ting rays of ultra violet irradiation cannot have been an important factor in the result. Extraneous protein must protect the virus in some other way. In this relation much importance attaches to the finding that the percentage inactivation of the virus in purified preparations by a constant amount of irradiation is dependent on the concentration of virus, the percentage destruction increasing as the concentration of virus was decreased by dilution. If the molecular ionization or excitation resulting from the high energy secondary electron occurred in the virus molecule itself, as postulated for other large molecular weight substances,—vaccine virus (14) and bacteriophage (15), for example,—because of the observed exponential relationship between inactivation and dose, then why should extraneous protein or virus concentration influence the percentage inactivation of the virus? It might be assumed that the presence of extraneous protein or of large numbers of virus molecules stabilizes the ionized virus molecule in some unknown way after it has been "hit," perhaps by some process analogous to the quenching of fluorescence. But this requires additional assumptions for which a factual basis is lacking. The findings as a whole can be best explained by assuming that the inactivation of the virus by x rays, particularly in dilute and purified virus suspensions, does not result primarily from direct hits on virus particles, but comes about indirectly through chemical reaction with some other molecules, probably those of the water present in the virus suspension, which become ionized or excited through absorption of the x rays. On this interpretation the observed influence of dilution of the virus or of the removal of impurities is readily understandable. A limited number of reactive water molecules would be formed in a unit volume of material by a given dose of x rays and the greater the amount of virus or impurities present in the suspension and exposed together with the virus to the effects of these activated water molecules, the smaller the fraction of virus that would be inactivated. Fricke (16) has proposed such an explanation of the effects of γ rays on the relatively small molecular weight proteins, pepsin and trypsin (17), and Woodward (18) had made the same assumption in connection with her experiments on lipase. The recently reported experiments of Dale (19) on the x ray inactivation of carboxypeptidase at various dilutions provide convincing evidence for this mechanism of x ray action on enzymes. Our experiments indicate that such an interpretation will best explain the inactivation of serum antibody by x rays.

The findings raise the question whether indirect chemical reactions are important for the chromosomal or genetic effects produced by γ rays. Cells contain a high concentration of protein and other materials and it seems likely that the indirect x ray effects of activated water molecules would become less important as the concentration of the solution increased. But until these conditions can be defined both the direct and indirect mechanisms should be considered in the effects of γ rays on chromosomes and genes.

The x-rays did not alter the papilloma virus in any qualitative respect, while reducing or abolishing its infectivity. Such portion of it as remained pathogenic elicited characteristic papillomas and these only. The demonstration that a larger dose of x-ray is necessary to abolish the complement-binding capacity of the papilloma virus than to render it non-infectious accords with the findings with certain plant viruses (20) and provides another means of destroying the infectivity of virus suspensions without removing the capacity of the virus to act as an antigen.

SUMMARY

The pronounced resistance to the x-rays manifested by the papilloma virus in ordinary suspensions is due to the protecting influence of extraneous matter and also in considerable degree to the amount of virus present in the preparation. Two to 4 million r were required to inactivate the virus contained in the crude papilloma extracts prepared for the present work, whereas 100,000 r or less was enough to inactivate comparable concentrations of virus after extraneous matter had been excluded by repeated differential centrifugation. The addition of normal rabbit serum or crystalline egg albumin to purified suspensions of virus was found to increase greatly the amount of irradiation required to inactivate the virus. Furthermore the percentage destruction of virus by a given amount of irradiation increases as the concentration is decreased by dilution with saline or buffer solutions. As little as 3,000 r will inactivate much of the virus in very dilute suspensions. The complement-binding antigen of papilloma virus suspensions is also inactivated by x-rays, but requires a somewhat larger amount of irradiation than necessary to destroy the infectivity of the suspensions.

The effects of irradiation on the antiviral antibody present in the blood of animals which have become immune to the virus—an antibody that specifically fixes complement in mixture with the papilloma virus—are also conditioned by extraneous material. 250,000 to 500,000 r had only a slight effect on the antibody in whole serum, while this amount of irradiation completely inactivated comparable amounts of antibody in preparations partially purified by precipitation with ammonium sulfate.

As these findings indicate that under certain conditions of purity and concentration the effect of the radiation does not act by direct hits on virus or antibody but indirectly by ionizing or exciting some other molecules present in the suspension, which then react with the virus or antibody molecule.

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COMPARATIVE VIRULENCE OF ST LOUIS ENCEPHALITIS VIRUS CULTURED WITH BRAIN TISSUE FROM INNATELY SUSCEPTIBLE AND INNATELY RESISTANT MICE

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There is reason to believe that the response of normal mice to experimental inoculation of St. Louis encephalitis virus depends largely upon properties of the brain tissue itself. Thus, virus dropped into the nares of innately resistant mice reaches the brain as promptly as virus similarly instilled into closely related, yet innately susceptible mice (1-3). Again serum from innately resistant mice is identical with that from susceptible mice in having no deleterious effect upon the virus. The main point, however, is that virus reaching the brain of resistants, no matter by what route, fails to increase in quantity beyond a certain point at which it is still harmless to the animal, whereas in the brain of susceptibles it increases 10,000-fold in amount above the point reached in resistant animals and brings about widespread nerve cell destruction and death.

To gain further information on the behavior of this virus in the brain tissue of susceptible and resistant mice, we turned to tissue culture methods and compared the titres of virus in different combinations of tissue plus serum mixtures.

Technique

Culture media consisting of serum Tyrode solution plus tissue were prepared according to the methods of Rivers and Ward (4) and Lloyd Theiler, and Ricci (5) except that embryo mouse brains were employed rather than whole mouse or chick embryo.

Tyrode solution from stock was measured according to the needs of each experiment and to it serum was added in a proportion of 10 per cent. Three types of serum were used—normal rabbit, normal adult mouse of a genetically resistant strain and normal adult mouse of a closely related yet genetically susceptible strain (3). This 10 per cent serum Tyrode solution was then filtered through a Seitz pad and transferred in 40 cc quantities to 50 cc Erlenmeyer flasks. Brain tissue from embryo or 1-day-old mice of three types was prepared and added to the filtered serum Tyrode solution in flasks in the following manner. Embryos from pregnant females of the W. Swiss strain of mice were removed close to term, their brains enucleated, washed in saline and minced with scissors. Brains from 1-day-old genetically susceptible and resistant mice (3) were also prepared in the same manner. Enough serum Tyrode was added

to the minced brain to make a 33 per cent suspension 3 gtt or about 0.15 cc of this tissue suspension was added to each flask containing 4.0 cc of serum-Tyrode

The virus inoculum consisted originally of 1 per cent suspension of infected brain tissue of a mouse injected intracerebrally with St. Louis encephalitis virus. The flasks were incubated for 3 or 4 days at 37°C and 1 cc of the supernatant was then transferred to the next flask.

The cultures were carried in triplicate with suitable tests for bacterial contaminants.

The activity of the culture virus was measured after 3 to 4 days incubation by preparing serial dilutions of a centrifuged supernatant and injecting 0.03 cc of each intracerebrally into 3-weeks-old W-Swiss mice. Two, and occasionally four or six mice, were used for each dilution.

Under these conditions we have cultivated the St. Louis encephalitis virus successfully since early 1935.¹

EXPERIMENTS

The behavior of St. Louis encephalitis virus in culture media containing brain tissue from (a) susceptible and (b) resistant mice was compared in the following manner:

A control medium was set up consisting of Tyrode plus 10 per cent adult rabbit serum plus brain tissue from embryo W-Swiss mice near term. A "susceptible medium" was also prepared consisting of Tyrode plus 10 per cent pooled serum from adult susceptible mice and brain tissue from 1-day-old susceptible mice, and a "resistant medium" of Tyrode plus 10 per cent pooled serum from adult resistant mice and brain tissue from 1 day-old resistant mice.

Three flasks of each medium were inoculated with 1 cc of culture virus from a previous control mouse embryo passage. Following 3 or 4 days incubation, 1 cc from each flask was transferred to another flask of similar medium. At least eight serial passages were made and certain of these cultures were titrated for virus activity.

The comparative activity of St. Louis encephalitis virus in media containing resistant and susceptible sera and brain tissue is shown in Tables I and II. Table I records the duration of life of mice injected with eighth-passage materials. Mice dying of St. Louis encephalitis virus infection did so after 5 to 9 days with rare exceptions. Titration end-points were clear-cut. In this test, the Swiss embryo culture titred 0.03 cc of the 1:10,000 dilution or better, the susceptible mouse culture, 1:10,000, and the resistant mouse culture, 1:160 dilution, a 62-fold difference. Table II summarizes the results of four series of tests. It shows, first of all, unpredictable variations in titre from test to test but consistency as to relative titres. Thus, in series 4, the eighth-passage

¹Jerome T. Syverton and George P. Berry reported the successful cultivation of St. Louis encephalitis virus in *Science*, 1935, 82, 596.

tests were consistently tenfold lower than the fourth passage tests. Secondly, the tests show that titres of virus in embryo brains are generally higher than those in 1-day-old brains. Finally, and of greatest interest to the present study, is the fact that the virus in the resistant media in each of eight tests showed a titre 1/10th to 1/100th of that in the susceptible media.

The question next arose of whether the relatively low virus titre in the resistant media might possibly be due to the presence of resistant serum rather than to resistant brain tissue.

Culture media were set up and inoculated as in the previous experiment except that two additional types of media were added: one containing sera from susceptible adult mice plus brain tissue from resistant 1 day old mice, and the

TABLE I

Virulence of St. Louis Encephalitis Virus Cultured in Susceptible and Resistant Media
Series 1—8th Passage

Medium	Fate of mice injected with 0.03 cc. culture virus in dilution										Titre 0.03 cc. of dilution	Dif- fer- ence in titre
	1/10	1/20	1/40	1/80	1/100	1/160	1/320	1/640	1/1000	1/10000		
Rabbit serum—Swiss embryo brain	5/5	—	—	—	5/5	—	—	—	6/7	8/10	1/10000+	
Susceptible mouse serum and brain	5/5	—	—	—	4/5	—	—	—	7/7	8/8	1/10000	
Resistant mouse serum and brain	6/6	7/8	6/8	7/8	—	7/5	7/5	5/5	5/5	5/5	1/160	62

* Mouse dead of St. Louis encephalitis 5 days following injection

S = mouse survived 30 days following injection

other, sera from resistant and brain tissue from susceptible mice. The comparative titres of virus in these several media are shown in Table III.

As in previous tests, the virus titred higher in embryo brain than in brain cultures from newborn mice, and different tests showed different end points. Of chief concern, however, was the fact that virus cultured with susceptible brain showed similar titres, regardless of whether the serum came from susceptible or resistant mice and virus cultured with resistant brain showed similar titres regardless of whether the serum came from resistant or susceptible mice. The titre of virus in resistant brain cultures remained 1/10th to 1/100th as high as that in susceptible brain cultures. In all, seven comparative tests have been made with similar results.

* F. Howell Wright, who tested the variability of titre of St. Louis encephalitis virus during and between experiments in our laboratory, noted that between experiments titres often varied tenfold in either direction and occasionally one hundredfold but that the relation of titres of virus in different media remained consistent.

Satisfied that the superior multiplication of St. Louis encephalitis virus in the brain tissue of susceptibles as contrasted with that of resistants is due to differences in the brain tissue itself, regardless of whether the tests are carried out *in vivo* or *in vitro*, we next attempted to determine whether the difference in titre in tissue culture was due to stimulating factors in the susceptible brain or to inhibiting factors in the resistant brain. The following protocol is an example of one of these tests.

TABLE II

Virulence of St. Louis Encephalitis Virus Cultured in Susceptible and Resistant Media
Summary of Results

Series	Medium	4th passage		6th-8th passage	
		Titre 0.03 cc. of dilution	Differ- ence in titre	Titre 0.03 cc. of dilution	Differ- ence in titre
1	Rabbit serum—Swiss embryo brain	1 1,000	} 100	1 10,000+	} 62
	Susceptible mouse serum and brain	1 1,000		1 10,000	
	Resistant mouse serum and brain	1 10		1 160	
3	Rabbit serum—Swiss embryo brain	1 1,000	} 10	1 100,000	} 50+
	Susceptible mouse serum and brain	1 1,000		1 1,000	
	Resistant mouse serum and brain	1 100		<1 20	
4	Rabbit serum—Swiss embryo brain	1 10,000	} 100	1 1,000	} 100+
	Susceptible mouse serum and brain	1 1,000		1 100	
	Resistant mouse serum and brain	1 10		0	
5	Rabbit serum—Swiss embryo brain	1 100,000+	} 20	1 100,000	} 10
	Susceptible mouse serum and brain	1 10,000		1 10,000	
	Resistant mouse serum and brain	1 500		1 1,000	

Cultures were prepared as above with the addition of two more sets, one of susceptible mouse serum and susceptible brain tissue from newborn mice plus 0.5 cc. of the supernatant from centrifuged 10 per cent resistant brain tissue, and the other of resistant mouse serum and resistant newborn brain tissue plus 0.5 cc. of the supernatant from centrifuged 10 per cent susceptible brain tissue.

This procedure was planned to demonstrate a possibly enhancing effect of susceptible extract containing relatively few cells or an inhibiting effect of resistant extract. Table IV shows that virus in the susceptible media titred 1 10,000 and in susceptible media plus resistant extract, the same,—1 10,000. Virus in resistant media titred 1 500 and in resistant media plus susceptible extract, slightly higher,—1 1,000 to 1 5,000. This slight difference occurred on repetitions of the test but it remains of questionable significance.

SUMMARY

We find that St Louis encephalitis virus cultured in 10 per cent serum-Tyrode solution plus brain tissue from 1-day-old innately susceptible mice attains a higher titre than when cultured in a similar solution plus brain tissue from 1-day-old closely related, yet innately resistant mice. This difference in titre persists regardless of whether the serum comes from innately susceptible or resistant mice. The relatively high titre of virus in the susceptible media is not affected by the addition of an extract (not cell-free) from the resistant brain, the relatively low titre of the virus in the resistant media may possibly be slightly enhanced by the addition of an extract from the susceptible brain.

The findings as a whole show that the marked difference in the increase of St Louis encephalitis virus in the brain tissue of innately susceptible and resistant mice, on culture *in vitro*, is due to some difference in the brain tissue itself.

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THE SEROLOGICAL SPECIFICITY OF PARTICULATE COMPONENTS DERIVED FROM VARIOUS NORMAL MAMMALIAN ORGANS

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Particulate components of tissue cells sedimented by high speed centrifugation from filtrates of organ suspensions have been encountered during attempts to purify viruses present in certain infected organs (1-5). In a number of instances, normal tissues have been found to yield particulate matter remarkably similar to that derived from diseased cells. A considerable amount of evidence indicates that such particles derived from the lungs of mice infected with the virus of influenza A are carriers of the pathogenic agent (6).

Claude has pointed out the possible identity of such cellular components with mitochondria. Since these cytological constituents are believed to play a significant rôle in specific cellular metabolism and differentiation, the suggested mitochondrial origin of the isolated particles implies that they may possess specific serological properties. Some experiments demonstrating such serological specificity have been reported briefly (7). The present paper constitutes a more detailed and extended study of the subject.

Materials and Methods

Preparation of Normal Organ Particles—The several organs of ether killed Swiss mice were usually frozen at -72°C immediately after dissection and stored at -10 to -15°C . Organs from rats, rabbits and ferrets were obtained in similar manner while organs from hulls and pigs were secured from the abattoir shortly after the death of the animals and stored at -10 to -15°C immediately upon arrival at the laboratory. Human organs were taken from cases coming early to autopsy, only those organs without apparent damage being retained.

The organs were ground with sand and powdered Pyrex to form a 10 to 20 per cent suspension in saline buffered at approximately pH 7.0. After low speed centrifugation the supernatant fluid was either passed through a medium Mandler or Berkefeld

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filter or centrifuged at 4,000 to 5,000 R P M for 30 minutes. This was followed by repeated centrifugation (8) at 20,000 to 25,000 R P M for 20 minutes. The pellet obtained was resuspended in fresh buffered saline after each centrifugation, by means of a rubber-tipped plunger with a diameter slightly less than that of the tube. The washed material was then centrifuged at 1,500 R P M to remove any larger aggregates. Particles from the following organs were prepared: brain, kidney, liver, lung, heart muscle, pancreas, spleen, and testicle.

The material sedimentable from Berkefeld filtrates in each case formed a translucent, yellowish to reddish brown pellet of gelatinous consistency. Under dark field examination, the resuspended particles gave an impression of size uniformity and a diameter of 0.1 to 0.3 μ was indicated by considerations based on the physical constants of the filter and centrifuge.

The nitrogen content of the suspensions used as antigens was determined by means of turbidity measurements with a Klett-Summerson photoelectric colorimeter. 1 ml of the suspension was diluted with 4 ml of 0.1 N NaOH, and the turbidity was then determined using a blue number 42 filter. Calibration curves for each of the several kinds of organ suspensions were plotted giving the nitrogen content per milliliter against the turbidimeter reading. The procedure made possible rapid estimation of the N content of a suspension within about 10 per cent.

For the injection of rabbits, suspensions with approximately 0.01–0.1 mg N/ml were used, depending on the yield of particles from the various organs. For the agglutination tests, suspensions containing 1–2 mg N/ml were found to give the most satisfactory results, and for the complement fixation reaction the latter suspensions were used diluted serially beginning with a 1:5 or 1:10 dilution of the original antigen. Brain particle preparations contained only one-third as much N as the other concentrates.

Lipoid Extracts—For preparation of alcoholic extracts the bovine organs were passed through a meat grinder and dried from the frozen state after which approximately 20 ml of 95 per cent alcohol was added for each gram of dried material. After 48 hours at room temperature, the extract was filtered through paper. The final preparations of alcoholic extract contained 1 to 2 per cent of lipoid by weight. For serological tests, the extracted material was suspended in saline by diluting 1 ml of extract with 5 ml of saline added dropwise.

Ground and dried organs were extracted with ether in a similar manner, but in this case the extract was dried down in an evaporating dish and the residue emulsified in saline. A milky suspension resulted in both instances.

Preparation of Antisera—Rabbits of about 3 to 4 kilos weight were inoculated intravenously or intraabdominally with 2 ml amounts of the various suspensions at 2 to 3 day intervals. In some instances, five injections led to a satisfactory antibody response (liver, kidney), in others up to nine injections were necessary (lung, testicle). This may have resulted from the fact that the concentration of particles as determined by the amount of N per milliliter of suspension varied among the different preparations.

Intravenous injection of the organ particle suspensions led to rapid death in a number of rabbits, and autopsy usually revealed clotted blood in the large veins and in the right heart. Even intra-abdominal injection resulted in the loss of two rabbits,

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Intravenous injection of the organ particle suspensions led to rapid death in a number of rabbits, and autopsy usually revealed clotted blood in the large veins and in the right heart. Even intra-abdominal injection resulted in the loss of two rabbits,

although in these cases death did not occur immediately after the injection as in the case of intravenous administration. Experiments with mice showed that the toxicity could occasionally be neutralized by normal rabbit serum (9) and could be eliminated by a number of the particle antisera.

The rabbits were bled 8 or 9 days after the last injection and the sera stored at -10 to -15°C . Normal serum taken from the same rabbits before the injections were begun was also kept frozen for reference.

Agglutination Tests—For the slide agglutination reaction the inside of a large Petri dish (diameter 20 cm) was marked into 16 squares with a wax pencil. A drop of serum, usually diluted 1:1 with saline, was placed in each square and a drop of particle suspension added. After stirring each mixture with an individual tooth pick or wooden applicator the Petri dish was closed and inverted. Further agitation of the fluids was obtained by rotating the dish in the horizontal plane. Readings were made after 5, 10, and 20 minutes at room temperature and recorded in the usual notation: 0 = no agglutination; 4 = all particles clumped in one or two large patches; 1 to 3 = various degrees of clumping.

For the test tube agglutination serial dilutions of antiserum were made and equal amounts of the particle suspensions added to a total volume of 0.4 ml. Readings were made after 30 and 60 minutes at 37°C , although in some instances (kidney, liver) flocculation occurred almost immediately in the more concentrated serum. For the agglutination of red blood corpuscles a 1 per cent suspension of washed erythrocytes was used. The readings were graded in the usual way: 0 = no agglutination; 4 = complete agglutination with clear supernatant fluid; 1 to 3 = various degrees of agglutination.

Complement Fixation Reaction—Serial dilutions of the particle suspensions were made and equal amounts of diluted antiserum and complement were added (0.2 ml. of each). After a preliminary incubation for 1 hour at 37°C , 0.4 ml. of a 2.5 per cent suspension of sensitized sheep cells were added and readings were made after 20 and 60 minutes at 37°C . Two units of complement and 2 units of amboceptor were employed. The readings were graded as follows: 0 = no hemolysis; tr = trace of hemolysis; wk = weak; st = strong; ac = almost complete; c = complete hemolysis.

Absorption of Antisera—A sufficient amount of a suspension of particles was sedimented at 20,000 R.P.M. and the supernatant fluid carefully drained off. The sediment was resuspended in undiluted antiserum, incubated for 30 minutes at 37°C , left overnight in the refrigerator, centrifuged again at 20,000 R.P.M. for 20 minutes and the supernatant fluid used. A second absorption was usually not required.

EXPERIMENTAL

General Activity of the Antisera

The study of the general activity of the various antisera against organ particles revealed that precipitins for the serum of the species from which the particles were derived were absent, or present in small amounts only. Agglutinins for red blood corpuscles of the homologous species usually were not increased as compared with the control serum taken before immunization. In a few instances however, a rise in the normal red blood cell agglutinins was noted,

which in two anti-mouse liver sera appeared to be significant ($>$ eightfold). The sera against murine organ particles all hemolyzed sheep cells in the presence of complement, thus indicating the formation of Forssman antibodies. This observation confirmed that of Furth and Kabat (4) who worked with particles derived from the spleen of the chicken which, like the mouse, belongs to the Forssman group of animals. The Kahn test and the Wassermann reaction were positive in a number of rabbits, but sera taken from the same animals before immunization were also positive.

Tissue Specificity

Slide agglutination of various normal murine, bovine, and a few human organ particles by homologous and heterologous antisera gave essentially similar re-

TABLE I

Slide Agglutination of Bovine Organ Particles by Selected Homologous and Heterologous Antisera

Suspension of particles derived from	Rabbit serum (1:1) vs. particles of bovine						Normal sera*
	Brain	Kidney	Liver	Lung	Heart muscle	Testicle	
Brain	3	0	0	0	0	0	0
Kidney	0	4	0	1	0	0	0
Liver	0	0	4	0	0	0	0
Lung	0	0	0	2	0	0	0
Heart muscle	0	2	0	0	4	0	0
Testicle	0	0	0	0	0	3	0

* Includes all sera taken from the various rabbits before immunization was begun.

sults. Table I shows the results obtained with bovine particles and selected antisera against bovine organ particles. Few cross-reactions between the different organs were found. More overlapping was observed with other sera and in the slide agglutination reaction of murine particles (7). The crude organ suspensions tested against homologous particle antisera were rarely flocculated, and if so only to a slight degree.

Cross-reactions between the various particles and the corresponding antisera increased with the number of injections. This observation is in agreement with similar findings in other immunological fields. Furthermore, the age of the particle preparation appeared to affect the specificity of the results. As the time of storage in the refrigerator was increased more and more non-specific reactions were noted and auto-agglutination of the suspensions became apparent. Therefore, the particle preparations were tested shortly after their preparation was complete and were usually not used after 48 to 72 hours.

Two additional techniques were employed to establish more definitely the

specificity of the antibodies, i.e., serum titration and absorption of the sera with heterologous particles. Upon dilution of the serum the agglutination of homologous particles was still positive when the heterologous reactions had become negative (Table II). However, the difference in titer between the homologous and heterologous sera was not very striking and more convincing results were obtained with the absorption technique.

After absorption of the sera with heterologous particles a definite homologous reaction remained (Table III) although most of the cross reactions were removed, especially by liver and kidney preparations. Lung and heart particle suspensions did not absorb all the cross reacting antibodies, but, since no attempt at quantitative absorption was made, conclusions as to the significance of this finding would be premature. Complete absorption of the sheep cell hemol

TABLE II
Effect of Dilution of Antiserum on Cross Reactions

Serum dilution	Rabbit serum against particles of bovine				Normal serum	
	Kidney		Liver		Kidney particles	Liver particles
	Kidney particles	Liver particles	Kidney particles	Liver particles		
1:2	4	2	2	1	0	0
1:4	4	1	1	1	0	0
1:8	1	0	0	3	0	0
1:16	0	0	0	2	0	0
0	0	0	0	0	0	0

ysis did not remove or alter the cross agglutinations. This indicates that the cross reactions were not caused by interference of the forssman antigen. Similarly, the distribution of precipitins for serum, red blood cell agglutinins, and Wassermann antibodies in the various sera did not furnish an explanation for the cross reactions, since no correlation with these antibodies could be established.

Organ specific antibodies were obtained against particles from brain, kidney, lung, liver, and testicle. Reactions with particles derived from heart muscle were found to be specific with some preparations but not with all. The mouse spleen particle antisera reacted only with the spleen preparation but all the other antisera gave a similar degree of agglutination with spleen particles except that against brain particles (7). The only antisera that did not show any reactivity were those against murine pancreas suspensions. It may well be that the amount of antigen injected was insufficient in this case, since the yield of pancreas particles was necessarily limited. Pancreas from other species has not been studied as yet.

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Testicle	0	0	0	0	0	3	0

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ment recorded in Table IV are included in this simplified form in Table V. When the serum was serially diluted and a constant amount of particle suspension added, similar demonstrations of dominant organ specificity were obtained.

Cross-reactions again could be removed by absorption with heterologous particles. The high speed centrifugation involved in this process removed all

TABLE VI

Effect of Absorption of Antiserum with Organ Particles on the Specificity of the Complement Fixation Reaction

Suspension of particles derived from bovine	Serum absorbed with particles from	Highest dilution of particle suspension reacting with rabbit serum (1:10) vs. particles of bovine						Normal serum
		Brain	Heart muscle	Kidney	Liver	Lung	Testicle	
Brain	—	1:160	0	0	1:20	1:20	1:20	0
	Kidney	1:80	0	0	0	0	0	
	Liver	1:80	0	0	0	0	0	
Heart muscle	—	0	1:40	0	1:10	1:10	1:10	0
	Kidney	0	0	0	0	0	0	
	Liver	0	0	0	0	0	0	
Kidney	—	1:40	1:40	1:160	1:20	1:20	1:20	0
	Kidney	0	0	0	0	0	0	
	Liver	1:10	0	1:80	0	0	0	
Liver	—	1:10	1:20	1:20	1:160	1:20	1:20	0
	Kidney	0	0	0	1:80	0	0	
	Liver	0	0	0	0	0	0	
Lung	—	1:40	1:40	1:40	1:40	1:160	1:40	0
	Kidney	0	0	0	0	1:80	0	
	Liver	1:10	0	1:10	0	1:80	1:20	
Testicle	—	1:20	1:20	1:20	1:40	1:40	1:160	1:5
	Kidney	0	0	0	1:5	1:10	1:80	
	Liver	1:5	0	0	0	1:20	1:80	

traces of antigen-antibody complexes which otherwise would have interfered with the reaction by showing "anticomplementary" activity of the serum. Results obtained with the absorbed sera are given in Table VI. All or most of the cross-reactions were eliminated by absorption with kidney particles, and usually with liver particles, leaving a distinct homologous reaction in all the antisera except that against heart muscle. In the exceptional case, although the native serum reacted dominantly with the homologous antigen, all reactivity could be removed with either liver or kidney particles. The cross-reactions

between testicle and lung particles were apparently not quantitatively removed by the absorption with either kidney or liver particles

TABLE VII
Species Specificity of Particles Derived from Various Organs
(Slide Agglutination)

Rabbit serum vs particles derived from	Serum absorbed with particles of	Particulate antigen derived from						
		Bull	Mouse	Rat	Rabbit	Ferret	Pig	Man
I Kidney								
Beef kidney	—	4	0	0	0		0	
Mouse kidney	—	0	4	0	0		0	
Rat kidney*	—	0	0	2	0		0	
II Lung								
Beef lung	—	3	0	0	0	0	0	
Mouse lung	—	0	3	1	0	0	0	
III Testicle								
Beef testicle	—	4	0					
Mouse testicle	—	0	3					
IV Heart muscle								
Beef heart muscle	—	3	±		0			±
Mouse heart muscle	—	±	3		0			0
V Liver								
Beef liver	—	4	3	3	0	2	2	3
Mouse liver	Mouse liver	2	0	0	0	0	0	0
Mouse liver	—	3	4	3	0	2	2	1
Beef liver	Beef liver	0	3	1	0	0	0	0
Rat liver*	—	3	3	4	1	2	2	3
Beef liver	Beef liver	0	1	4	0	0	0	0
Rabbit liver*	—	0	0	0	1	0	0	0
VI Brain								
Beef brain	—	3	3	3	3		4	
Mouse brain	Mouse brain	±	0	0	0		0	
Mouse brain	—	2	2	2	1		2	
Beef brain	Beef brain	0	0	0	0		0	

* Antisera against whole organ suspension

Species Specificity

It was found that most of the particles showed species specificity in addition to the organ specific differentiation (Table VII). Antisera against bovine kidney agglutinated bovine kidney particles but did not react with kidney preparations derived from mice, rats, pigs, or rabbits. Similarly, antisera to lung, heart muscle, or testicle particles agglutinated only the preparations from the homologous species. There were, however, slight cross-reactions between mouse and rat preparations.

On the other hand, brain antisera reacted with brain particles of all the

different species tested, even the rabbit, in which the antiserum was produced. Absorption of the brain antisera with heterologous brain particles removed not only the cross-reactions with all brain preparations from heterologous species but also most or all of the activity with brain particles of the homologous species. Antisera against liver particles, likewise, showed a rather broad specificity, *i e*, antiserum to bull liver agglutinated to various degrees liver preparations from all the different species except the rabbit, which animal was used for the antibody production. However, absorption with liver preparations from heterologous species left a strong agglutination of liver particles from the homologous species but removed all cross-reactions.

When the complement fixation technique was used with higher dilutions of serum, cross-reactions were observed with liver preparations from a few species, but not with all. It appears that the liver particles are dominantly species specific but a small fraction of antigen common to all species is likewise present.

The complement fixation test occasionally gave cross-reactions with heterologous organs from various species. Such reactions were not observed in the agglutination test which appears, therefore, to be more specific under the conditions of these experiments.

Iso-Immunization

Some rabbit antisera against whole liver suspension showed a slightly different reactivity as compared with liver particle antisera in that they also slightly agglutinated rabbit liver particles. One rabbit whole liver antiserum agglutinated only rabbit liver particles to a slight degree (Table VII). The specificity of this reaction has not been studied. Attempts at iso-immunization with particles derived from various rabbit organs have been made. However, no iso-antibodies to particles derived from liver, kidney, lung, and brain have been noted after as many as 19 injections, while hetero-antibodies were formed after 5 to 10 injections.

Some Properties of the Antigens Involved

The antigens reacting in the complement fixation test with the various homologous and heterologous antisera are all heat-labile with the exception of the brain suspensions, *i e*, they are destroyed by heating the suspensions to 100°C for several minutes. Brain particle suspensions, on the other hand, withstood heating for 20 minutes without apparent loss of serological activity.

Since alcohol-soluble substances have been found to be responsible for organ specific reactions in a number of instances (10, 11, 12, and others) the antisera against bovine organ particles were studied for their content of lipid antibodies. Antisera against particles derived from brain reacted with alcohol or ether extracts of brain, and no cross-reactions were observed with extracts derived from other organs. None of the other sera contained significant amounts

of lipid antibodies, although some slight fixation of complement occurred in the highest concentration of extract used (Table VIII)

The relationship of this alcohol extractable antigen to the cross reacting substance in brain particle suspensions was studied by the absorption tech-

TABLE VIII

Complement Fixation Reaction with Alcoholic Organ Extracts and Various Antisera against Particulate Antigens

Alcoholic extract of bovine	Highest dilution of extract reacting with rabbit serum (1:10) vs particulate antigen of bovine					
	Brain	Heart	Kidney	Liver	Lung	Testicle
Brain	1:1280	0	0	0	0	0
Heart	1:5	0	0	0	0	<1:5*
Kidney	0	0	0	0	0	<1:5
Liver	1:5	0	0	0	0	<1:5
Testicle	0	0	0	0	0	<1:5

* Partial fixation of complement in this dilution

TABLE IX

Relationship between Alcohol-Soluble and Heat Stable Organ Specific Antigens in Particles Derived from Brain

Rabbit serum vs particles derived from	Serum absorbed with	Highest dilution of antigen giving complete fixation of complement			
		Particles derived from brain of			Alcoholic extract of bovine brain
		Bull		Mouse	
		Native	20-100°C.		
Bull brain (1:10)	—	1:320	1:320	1:160	1:1280
	Mouse brain particles	1:10	0	0	
	Alcoholic beef brain extract	1:80	1:80	1:40	0
Mouse brain (1:10)	—	1:160	1:80	1:80	1:160
	Beef brain particles	0	0	0	
	Alcoholic beef brain extract	1:80	1:40	1:40	0

nique Table IX shows that (a) after absorption with heterologous brain particles, the homologous as well as the heterologous reaction was removed and (b) after absorption with alcoholic extracts, a strong reaction with homologous and heterologous brain particles remained not only against native suspensions but also against heated preparations. There are, therefore, at least two cross reacting brain antigens present in these preparations, one extractable by cold alcohol or ether, the other not extractable by these solvents, but like-

wise heat-stable In addition, a few sera showed a small amount of species specific antibodies

DISCUSSION

The chemical nature and cytological identity of particles derived from normal organ filtrates by high speed centrifugation is under investigation, and the evidence that they may be mitochondria is strong The chemical composition (1) and staining properties, particularly by Janus green B (7) are in favor of such a view The serological specificity found in this study adds further support since the rôle usually ascribed to mitochondria in cellular differentiation and metabolism implies organ specific structure

The most specific serological results were obtained by the agglutination reaction Particles from all the organs tested revealed a certain organ specificity with the exception of those from the pancreas, in which case the amount of antigen injected probably was insufficient for the production of antibodies The particles were differentiated by an earlier, and usually stronger, agglutination in the homologous than in the heterologous antisera Absorption of the sera with heterologous particles left the organ specific agglutinins In addition, all particles except those derived from brain exhibited species specificity Some cross-reactions were observed between the liver particle preparations from various species but particles from the homologous species were generally agglutinated first, and absorption with liver particles from a heterologous species did not abolish the homologous reactivity of the serum Brain particle reactions, on the other hand, were independent of the species Absorption of the brain particle antisera with heterologous brain particles removed all or most of the reactivity, including that due to brain particles from the homologous species

The results of the complement fixation reaction with the particle concentrates resembled those reported in the literature for whole organ suspensions or extracts thereof Early attempts to demonstrate organ specific structures in suspensions of various organs yielded more or less indefinite results, with the well known exception of the lens of the eye (*cf* 13) More recently, Witebsky and his collaborators have studied the serological specificity of a number of different organs and lipid extracts thereof While brain suspensions exhibited a clear-cut organ specific structure comparable to that of the lens in that it was independent of the species (12), parenchymatous organs such as the liver and kidney showed only occasionally organ specific dominance and the reactions were at the same time limited to the species (12, 14) Suspensions of kidney were studied by Landsteiner and van der Scheer (15) and Hahn (16), preparations of testicle by Lewis (17) and Krupe (18) Using the globulin fraction of organ suspensions, organ specific antibodies were demonstrated for the thyroid gland by Hektoen, Fo τ , and Schulhof (19) and for preparations of

pancreas and adrenals by Witelsky (20) Also Wolff (21) was able to differentiate between proteins of kidney and liver by serological means using especially prepared antigens free of lipoids In most of these studies, cross reactions were quite marked and the demonstration of organ specific antibodies sometimes obscured

Also, in the present study, involving particulate fractions of the various organs, more cross-reactions were encountered in the complement fixation test than in the agglutination reaction but the particulate nature of the antigen made it possible to use absorption technique to advantage Traces of antigen antibody complexes left in the absorbed serum were removed completely by high speed centrifugation and so did not interfere in subsequent tests

The importance of alcohol-extractable substances in the serological specificity of organs has been demonstrated by Landsteiner and van der Scheer (10), Witelsky (11), Witelsky and Steinfeld (12), and others The particles from normal organs studied by us possessed a relatively large percentage of lipoids (12 to 20 per cent) However, antibodies to these alcohol or ether extractable materials were formed only in the case of brain particles which were found to be strictly organ specific but not species specific The lack of response to the lipid fractions of whole organ suspensions in rabbits was observed by Witelsky who studied the availability of lipoids as antigens in such mixtures (22) He found that the injection of suspensions of brain, lens, and certain tumors evoked lipid antibodies, while other normal organ suspensions failed to do so

The serological reactivity of alcoholic extracts of brain (12) agrees with the observation that only the particles derived from this organ withstood heating to 100° C for 20 minutes without much loss of reactivity The reactivity, however, was not entirely due to this lipid fraction, since the absorption of the brain antisera with alcoholic brain extract left a distinct reactivity of the serum with heated brain particle suspensions Two antigenic components are, therefore, present in these preparations, both independent of the species One is soluble in cold alcohol or ether, while the other is not extractable by these solvents Both are heat stable The relationship of this second antigen to the protagon fraction described by Sachs and Schwab (23) or to the antigen present in brain infusion broth as studied by Bailey and Gardner (24) has not been elucidated The outstanding behavior of the brain particles, however in addition to the low nitrogen content of these suspensions in contrast to other organ preparations, makes it questionable whether or not we deal with the same type of material in the two different groups

In the present study we have dealt with organ specific and species specific antigenic properties of particles from certain organs, and with combinations of the two, *i.e.*, species and organ specific antigens The need for adequate terminology to describe such structures is already apparent, and will doubtless become pressing when the studies have been extended to broader groups such

as the various classes of vertebrates, and the animal phyla. Various types of organ specificity have been enumerated by Witebsky (25)

The question of iso-immunization will also be of interest. Attempts to immunize rabbits with particles derived from various rabbit organs have not yielded positive results. However, it should be pointed out that it is not possible to iso-immunize with whole organ suspensions (12) unless they are altered by certain procedures (26, 27). Suspensions of spermatozoa constitute an exception (see 28).

Preliminary studies to determine the presence of possible nephrotoxic substances in the rabbit *vs* mouse kidney particle sera have yielded negative results thus far. The question whether or not cytotoxic substances may be present in other antisera remains to be investigated.

The relationship of the particles studied in this report to certain virus diseases is under investigation in various laboratories. The use of specific antisera against normal organ particles in attempts at purification of viruses from organ suspensions was considered a possibility, but, in the case of the virus of influenza A, present in mouse lungs, such a procedure was found to be ineffective (6). The normal mouse lung particle antisera agglutinated particles derived from infected lungs to a high degree and subsequent titration of the virus in the supernatant fluid and the agglutinate revealed that most of the virus was present in the latter. This indicated the carrier rôle of the lung particles for the virus of influenza A and further evidence pertaining to this point will be presented elsewhere.

SUMMARY

1. Particles derived from filtrates of organ suspensions by high speed centrifugation were serologically active as shown by agglutination and complement fixation techniques. Particles from brain, liver, lung, kidney, heart muscle, spleen, testicle, and pancreas of various species have been studied.

2. All particles showed a certain degree of organ specificity with the exception of pancreas. Cross-reactions occurred between the particles from various organs from one species, which were more marked when complement fixation technique was employed than by the agglutination test. However, agglutination always appeared earlier and was stronger, and complement fixation was positive in higher dilutions of antigen in the presence of homologous antiserum than with heterologous antisera.

3. The cross-reactions did not depend on the occasional precipitins for serum and the agglutinins for the red cells of the species from which the particles were derived, nor did they bear a relation to Wassermann and Forssman antibodies present in some of the sera.

4. The organ specific differentiation of the particles from various organs could more clearly be demonstrated by two means. The antiserum could be

diluted in such a way that only the homologous reaction still showed a positive result while the cross-reactions had become negative, or the cross reacting antibodies could be absorbed by heterologous particles and the homologous reaction was still more or less intact

5 In addition to the organ specific differentiation, most particles were found to exhibit species specificity. While the particles derived from kidney, lung, testicle, and heart muscle aggregated only in the presence of the antiserum against the corresponding organ particles from the homologous species, brain particles reacted with brain antisera against both homologous and heterologous species alike. Absorption of brain particle antisera with brain preparations from a heterologous species removed all antibodies. Liver particle preparations showed an intermediate position in that all liver preparations with the exception of rabbit liver particles were aggregated by any liver particle antiserum. However, absorption with liver particles from a heterologous species left a distinct species specific reaction in the serum.

6 The antigens involved are all destroyed by heating to 100° C for a few minutes with the exception of brain particles, which after 20 minutes at 100° C still gave complement fixation almost to the same strength as the untreated controls.

7 Alcoholic and ether extracts of brain reacted with the brain particle antisera only. All alcoholic or ether extracts of other organs gave no complement fixation. None of the various other organ particle antisera tested contained antibodies for these extracts.

8 The relationship between the heat stable and the alcohol soluble brain particle antigen studied by absorption technique revealed that there were two antigens present, both organ specific and independent of the species: the one alcohol and ether soluble, the other not soluble in these solvents but heat stable. Some of the sera showed besides a few species specific antibodies.

9 Preliminary evidence has been gathered to show that no isoimmunization could be obtained with any one of the organ particles. As far as cytotoxic activity of the sera is concerned only the kidney particle antisera have been studied for nephrotoxins, these failed to reveal any such activity in the mouse.

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STUDIES ON REACTIONS RELATING TO CARBOHYDRATES AND POLYSACCHARIDES

LXIV ANTIGENICITY OF DEXTRAN PRODUCED BY *LEUCONOSTOC MESAENTEROIDES*

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Immunological studies on dextran (Zozaya, 1932, and FitzGerald, 1933), a glucose polysaccharide produced by the action of *Leuconostoc mesenteroides*, have been directed towards its possible properties as an antigen. In this investigation, however, an attempt has been made to show that dextran possesses the properties of a haptene, that is, to prove a pure polysaccharide can give a precipitin reaction with anti *Leuconostoc* sera prepared with the homologous organisms.

Recently, Hehre (1941) has reported the production from sucrose of an antigenic polysaccharide by living cultures of *L. mesenteroides* and also by an active principle contained in sterile, filtered extracts from these same cultures. This polysaccharide, which is believed to be identical with the dextran described by Fowler, Buckland, Brauns, and Hibbert (1937), reacted with antisera of Types II and V of pneumococci and with the homologous antiserum of the bacteria. Apparently this anti *Leuconostoc* serum was prepared by inoculation of rabbits with intact bacteria, but no details of its preparation are given. The dextran, which gave a reaction with the homologous antiserum, was not isolated or subjected to purification in order to remove other antigenic components.

Methods and Results

Preparation and Titration of Anti Leuconostoc Sera—Large scale production of *L. mesenteroides* culture No. 5 (Hucker and Pederson, 1930) was carried out using the medium of Tarr and Hibbert (1931) containing only 0.25 per cent sucrose and having the peptone solution of original pH 7.2. This medium was inoculated with a 24 to 48 hour culture and it was found that the addition of 1 per cent tomato juice (prepared from canned tomatoes) was necessary for growth of the organisms. Incubation was allowed to proceed for 3 days at room temperature (approximately 25°) and all precautions were taken to prevent, and ensure absence of, contamination. Sufficient formalin was then added to give a 1 per cent solution, and the culture was stored at 0° for at least 18 hours. The organisms were separated by centrifuging in a

large angle-centrifuge, observing sterile precautions. The bacterial precipitates were washed twice by centrifuging with 0.1 per cent formalinized saline, and some (called Antigen B below) were heated at 80° for ½ hour. Other suspensions (Antigen A) were not subjected to this heat treatment. The organisms were examined, and found to be still Gram positive, and capsulated. The suspensions were then standardized against Brown's (1919) tubes, using pneumococcus values as comparison, since no values were available for *Leuconostoc* suspensions. The concentrations of suspensions prepared were $2000 \times 10^6/\text{cc}$ and $400 \times 10^6/\text{cc}$. Rabbits were inoculated intravenously in the marginal vein of the ear, following an immunization schedule similar to that used by Goodner, Horsfall, and Dubos (1937) for the production of antipneumococcus sera. Rabbits 1 and 2 were duplicates inoculated with Antigen A, rabbits 3 and 4, also duplicates, inoculated with Antigen B.

The immunization schedule involved doubling the numbers of organisms in successive injections for 4 days, then rest for 3 days, then inoculations for 4 days, rest, etc., until sufficiently high antisera titres resulted. During the first 2 weeks of the schedule, the injections were increased from 0.1 cc 400×10^6 organisms per cc to 0.4 cc 2000×10^6 organisms per cc. Inoculations were again commenced at the 10th week, and increased to 0.4 cc 2000×10^6 organisms per cc by the middle of the 12th week. The rabbits were bled after rest for a further 10 days, and the antisera titrated. Reinoculation for 4 days, followed by rest for 10 days, then bleeding and titration of sera, then reinoculation (to a maximum of 1.0 cc 2000×10^6 organisms per cc) etc., was continued until the final bleeding at the 19th week of the immunization schedule.

Rabbits 5 and 6 were inoculated during the same period with Antigen B, but a 3 or 4 day rest was allowed between each inoculation. Work with these rabbits was discontinued, since the agglutination titres of the antisera were not as high as those of rabbits 1-4.

Large-scale bleedings were carried out by cardiac puncture, small-scale, from the ear. The blood was allowed to clot for 24 hours at room temperature, then the cells were separated by centrifuging. The serum was decanted, sterilized by heating at 55° for ½ hour, then stored at 0° in serum vials with 1:10,000 merthiolate as preservative.

The agglutination titres increased during the inoculation schedule. Table I shows these values at the end of the inoculation schedule.

The agglutination reactions were carried out at 55° for 6 hours, and the final readings were made after a further 12 to 18 hours at room temperature. The concentration of antigen used was 4×10^8 organisms per cc.

Preparation of Dextran for Precipitin Reactions—Dextran was prepared using the medium of Tarr and Hibbert (1931), modified by the addition of 1 per cent tomato juice, and 0.5 per cent calcium carbonate. The addition of calcium carbonate was found to increase the yields of dextran obtained, probably by neutralizing the acid produced, thereby maintaining the reaction of the culture about pH 6, and permitting a longer period of dextran production. Incubation was allowed to proceed for 15 to 20 days at 25°. The culture solution was then evaporated to about one-fifth its volume in vacuum, and in an atmosphere of nitrogen (bath temperature below 50°) and the solution maintained about pH 7. The crude dextran was precipitated by pouring the viscous concentrate into three volumes of methanol, washed by grinding

with methanol and ether, and then dried in the air and finally under vacuum at 50°. The yields of dextran, in the form of a fine, white powder, averaged 25 gm per litre of culture

TABLE I
Titration Anti Leuconostoc Sera (19th Week)

Antibody dilution	20	40	80	160	320	640	1280	2560	5120	10 240
Rabbit No										
1		++++	++++	+++(+)	+++	+++	++	+	(+)	T
2		++++	++++	+++(+)	+++	++	+(+)	+	(+)	T
3	++++	++++	+++	+++	++	+	(+)	T	T	t
4		++++	++++	++++	+++	++	+	+	(+)	T

Antibody and antigen controls were negative

++++ Large flocculations complete agglutination

+++ Small

++ Large particles in suspension

+ Small

T Turbidity (probably due to a precipitin reaction of the polysaccharide)

t Slight turbidity

++(+) e.g. intermediate between +++ and ++

TABLE II
Analyses of Dextran Preparations Used as Antigens in the Precipitin Reaction
Calculated for $(C_6H_{10}O_5)_x$ C 44.4 H 6.17

Preparation	C	H	N	Ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A†	43.8	6.46	0.09 0.10 0.10 0.11	0.6
B	44.4	6.37	0.09 0.10 0.10 0.11	0.23
C	44.1	6.55	0.033 0.078	0.15
D (crude dextran)			0.16 0.16	0.5

* All these Kjeldahl nitrogen determinations were carried out using N/100 standard acid and base. Analyses of A and B were carried out in a standard micro apparatus using 50 to 70 mg samples. Analyses of C and D were carried out in a macro apparatus using 0.2 gm samples for C, and 0.1 gm samples for D.

† This sample was prepared from a different lot of the crude dextran (D) and only reprecipitated once.

The crude dextran which contained sucrose was purified by dialysis against flowing tap water (700 cc per minute 37°) for 8 hours using Du Pont No. 300 cellophane as the membrane. The dextran was precipitated as described above, yielding about 7 gm from 10 gm of the crude product. The purified product gave negative protein (biuret and xanthoproteic) and nitrogen tests, and was qualitatively free of reducing

TABLE III
Precipitin Titrations with Anti *Leuconostoc* Sera and Dextran

Rough titrations		Dilution of antigen/100												
B and dilution	G	1	2	4	8	16	32	64	128	256	512	1021	2018	4096
No 2	1 6 A		-		-	++(+)	++	++	++	++(+)	+	(+)	-	-
	1 2 A		-	+	++	++	++	++	++	++(+)	(+)	-	-	-
	1 2 B		++	++	++	++	++	++	++	++(+)	++	-	-	-
	1 2 C	++	++	++	++(+)	++	++	++(+)	++	++	++	+	-	-
	1 2 D	++	++	++	++	++	++	++	++(+)	++	++	(+)	-	-
No 11	1 2 A		-	+	++(+)	++	++	++	++	++(+)	++	++	++	++
	1 2 B		++	++	++	++	++	++	++	++(+)	++	++	++	++
	1 2 C	-	-	+	++	++	++	++	++	++	++	++	++	++
	1 2 D		-	++	++	++	++	++	++	++	++	++	++	++
No 1	1 2 A		-		+	++	++	++	++	-	-	-	-	-
	1 2 D		++	++	++	++	++	++	++	++	++	++	++	++
No 3	1 2 A		-		++	++	++	++	++	(+)	-	-	-	-
	1 2 D		++	++	++	++	++	++	++	++	++	++	++	++
No 4	3 4 A		++	++	++	++	++	++	++	++	++	++	++	++
	1 2 D		++	++	++	++	++	++	++	++	++	++	++	++

B, antisera G, dextran preparations used as antigens Bold faced signs indicate optimal proportion tubes No 11 from No 1 rabbit at 17th week of immunization schedule Controls were negative
 ++++ Complete precipitation, no suspended particles
 +++ Precipitation, and large
 ++ Large suspended particles, no precipitation
 + Small
 ++ e.g. intermediate between ++ and +

sugars and sucrose. It contained 0.5 per cent ash, (Preparation D, Table II) and was subjected to varying degrees of purification as follows.

(a) *Preparation A* By electrodialysis of a dextran solution (4 gm Preparation D in approximately 150 cc) in a small Pauli apparatus at 110 v against distilled water (temperature below 30° for approximately 60 hours, pH adjusted at intervals to approximately 7). The dialyzed solution was filtered, concentrated to approximately 50 cc and precipitated into methanol. The analyses of this product are shown in Table II.

(b) *Preparation B* Electrodialyzed a total of 84 hours, as with Preparation A, but against 0.4 per cent sodium hydroxide, (Fowler, Buckland, Brauns, and Hibbert, 1937). In the first hour or two of such electrodialysis, the temperature rose to about 90° but replacing of the sodium hydroxide solution with water brought about a temperature drop to room temperature. The distilled water was renewed at intervals, and another short dialysis period against 1 per cent sodium hydroxide was

TABLE IV
Precipitin Titrations with Anti-Leuconostoc Sera and Dextran

Fine titrations

Antibody and dilution		G	Dilutions of antigens (G)/100												Optimal ratio B/G	
No 2	1	A	30	40	50	60	67	75	86	100	109	120	134	150	2900	(8600/3)
	3	B	16	20	24	27	30	34	37	40	44	48	53	60	1100	(3400/3)
	2	C	25	30	33	37	43	46	50	54	60	67	75	90	2200	
	3	D	8	10	12	13	15	17	20	24	27	30	34	40	800	
No 1F	1	A	20	25	30	35	40	45	50						1500	
	2	B	16	20	24	27	30	34	37	40	44	48	53	60	1700	
	2	C	30	33	37	43	46	50	54	60	67	75	90		2700	
	2	D	8	10	12	13	15	17	20	24	27	30	34	40	850	

Bold faced numerals indicate optimal proportion tubes

carried out, then against distilled water until the current was approximately zero for some time. The product was recovered in the usual manner and precipitated into methanol three times. Analyses of this product are shown in Table II.

(c) *Preparation C* Identical with Preparation B, except electrodialysis was carried out against 1 per cent sodium hydroxide (renewed four times) for approximately 2 weeks. The product was reprecipitated three times. Analyses of this product are also shown in Table II.

Precipitin Reactions—The antisera used were the anti *Leuconostoc* sera described above. With dextran Preparation D (Table II), dilutions varying from 1/100 to 1/100,000, and antibody dilutions about 1/15 slight precipitation was observed in the antiserum from rabbit No. 2 only. Precipitation occurred, however, in higher concentrations of these antisera as shown in Table III. The results were read after 16 to 24 hours at room temperature. Fine titrations are shown in Table IV.

DISCUSSION

For the preparation of anti *Leuconostoc* sera, several different inoculation schedules were tried, and slightly different antigens used. Formalin was added

to all *Leuconostoc* suspensions used as antigens to obviate enzymatic changes, loss of Gram-positive character, etc (Goodner, Horsfall, and Dubos, 1937) In addition Antigen B was heated at 80° for ½ hour, and the titres of sera produced by this antigen (rabbits 3 and 4) were lower than those produced by Antigen A (rabbits 1 and 2) which was not heated in this manner Lower titres of antisera were also obtained when rest periods of 3 to 4 days were allowed between each inoculation (rabbits 5 and 6) rather than daily inoculations (rabbits 1, 2, 3, 4) for 4 days, followed by a rest period for the remainder of the week In both these cases, however, the number of rabbits was too few to make these generalizations regarding low titres absolutely conclusive

Early work (FitzGerald, 1933) has shown that dextran is incapable of production of antibodies when the nitrogen content of the polysaccharide is reduced below 0.2 per cent Zozaya (1932) has shown that, by absorption on collodion, dextran may function as an antigen, the inference being drawn that this immunological activity is due to the colloidal nature of the dextran-collodion complex

The conclusions, reached in previous work, however, cannot be accepted without reservation since there is some doubt whether the dextran preparations employed were truly nitrogen-free Although these dextran preparations gave a negative qualitative nitrogen test, no highly sensitive quantitative analysis was made

In this investigation, an attempt has been made to establish the fact that nitrogen-free dextran, while it may not be a true antigen, can function as a haptene, that is, give a precipitin reaction with anti-*Leuconostoc* sera prepared with the homologous organisms Considerable effort has been directed toward purification of the dextran used in the precipitin reactions in an attempt to remove the last traces of nitrogen Preparations of purified dextran have been shown to give negative qualitative nitrogen and protein tests However, the reliability of such tests as used (*e.g.* Heidelberger, Kendall, and Scherp (1936)) in the presence of large amounts of carbohydrate is questionable since the presence of carbohydrate appears to reduce their sensitivity This conclusion has been borne out by the fact that small amounts of casein when mixed with starch cannot be detected by qualitative nitrogen or protein tests

In order to detect the smallest percentage of nitrogen which could possibly be present in the dextran preparations, used in this investigation, a highly sensitive, quantitative nitrogen analysis procedure has been developed This is a modification of the Kjeldahl method in which the usual macro sample (*ca.* 200 mg) is used and titrations made with \approx 1/100 standard acid and base In this way sensitivity of the normal Kjeldahl analysis has been considerably increased The results, reported for the purest dextran preparation (C, Table II), indicate a nitrogen content of 0.08 per cent It should be pointed

out, however, that this value represents the *maximum* nitrogen content since a small blank sometimes results from analytically pure carbohydrates. The nitrogen content of each of the purified dextrans is lower than that usually reported for pneumococcus polysaccharides to which immunological activity is ascribed (Heidelberger, Kendall, and Scherp, 1936, and Brown, 1939). The purest dextran preparation (C, Table II) could have, as a maximum, only 0.5 per cent protein based on the nitrogen analysis. The nitrogenous impurity in the dextran might be peptone, which would not in itself influence immunological reactions, or bacterial nucleoprotein, or the nitrogen might be an integral part of the polysaccharide molecule.

The precipitin reactions, given by the various preparations of dextran, show that as the nitrogen content is decreased (compare Preparations C and D, Table II), precipitation takes place at a higher dilution. It seems apparent from this relationship that the antigenic activity of the dextran is not dependent on traces of nitrogenous impurity since the small amount of nitrogen (0.1 to 0.2 per cent) would not be expected to cause the observed variations in titres given by the various preparations of dextran (Table III). Deflection of the optimal proportions ratio (Table IV) during the various stages of purification is not clear. This may be due to cleavage of the polysaccharide chain as a result of contact with alkali during purification. Such cleavage might be expected to give rise to an increase in the number of antigenic units in a given weight of dextran. An attempt has been made to detect cleavage of the polysaccharide chain by viscosity measurements of the various dextran preparations, but these results are inconclusive since the specific viscosities varied over a wide range when determined at different concentrations.

SUMMARY

Anti *Leuconostoc mesenteroides* sera have been produced in rabbits. These antisera gave precipitin reactions with relatively high dilutions of the homologous polysaccharide dextran, having a maximum nitrogen content of 0.08 per cent. It can therefore be concluded that this dextran is a haptene.

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THE NATURAL HISTORY OF HUMAN POLIOMYELITIS

II ELIMINATION OF THE VIRUS*

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The purpose of the present investigation was twofold (1) to determine by which secretions and excretions *infective* poliomyelitis virus is chiefly eliminated from the human body, and (2) to discover, if possible the origin of the eliminated virus. This study which was to be carried out on living poliomyelitis patients was planned simultaneously with another investigation the purpose of which was to map the distribution of the virus in the body after death (1). The results of this latter task have been reported (1). At the time the present study was undertaken, these results were not yet available but the following was known about the elimination of virus in human poliomyelitis (1) that in several hundred tests recorded in the literature the virus had been demonstrated on a number of occasions in nasopharyngeal washings, although the manner in which the tests were performed failed to indicate whether the virus originated from the nose or from the mouth and pharynx or both (2) that the virus had been found in the oro-pharyngeal washings on two occasions during the first 2 days of illness in abortive poliomyelitis although similar tests on a few patients with paralytic poliomyelitis were negative (2), (3) that compared with its rare occurrence in nasopharyngeal washings, the virus could be isolated with considerable frequency if not regularly from the stools of patients with abortive, non paralytic, and paralytic poliomyelitis, and "healthy" contacts as well (3-6).

The commonest explanation for the presence of the virus in the stools had been that it had its origin in the swallowed secretions of the upper respiratory tract. The greater incidence of positive isolations from the stools was accounted for by the fact that virus swallowed over a period of days could be concentrated in the colon. When it was recalled that the nasopharyngeal washings represented at best only a casual sample of the secretions, this explanation did not lack plausibility and it appeared possible that if these secretions were collected over a period of days the virus might be demonstrated in them with as great frequency as in the stools. In order to test this hypothesis,

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and without any preconceived plan of separating the nasal from the pharyngeal and oral secretions, the nasal secretions were collected by alternately plugging one or the other side of the nose with absorbent cotton for a period of 3 days, at the end of this period a stool or enema returns specimen was obtained to serve as an index to the presumable accumulation of virus in the lower intestine during the interval under investigation. Whenever possible, and that, unfortunately, was usually only among the older patients, the oral secretions and saliva were also collected during the same period. The urine of a number of patients, particularly when clinical signs of bladder involvement were present, was studied to test the hypothesis that poliomyelitis virus may spread centrifugally from the spinal cord along certain nervous pathways to the intestines and urinary bladder and ultimately be eliminated from these sites.

Collection of Specimens and Methods of Preparation

The material for this study was collected in the summer of 1940 from patients in Jackson, Michigan, Indianapolis, and Cincinnati¹

Nasal Secretions—Absorbent cotton was plugged alternately into one or the other side of the nose every 6 to 18 hours for a period of 3 days. As each plug was removed (usually soaked with secretions) it was stored in a refrigerator. At the end of the 3 days, the 6 plugs usually obtained from each patient were pooled and transported to the laboratory in the frozen state (solid CO₂ was used). The secretions were expressed from the plugs in several steps. (1) approximately 4 to 7 cc of undiluted secretion was obtained by compressing the plugs in a syringe containing a piece of wire gauze over its floor, this specimen was centrifuged at about 2,000 R P M for 10 minutes, the sediment being saved for nasal instillation while the supernatant liquid after etherization was used in part for intracerebral inoculation and the remainder combined with the second extract for intraperitoneal inoculation, (2) the second extract was obtained by macerating the plugs in 5 to 10 cc of physiological salt solution and again expressing the fluid with the aid of a syringe, after centrifugation, as above, the sediment was saved for nasal instillation, and the supernatant liquid, after etherization, for intraperitoneal inoculation, (3) the fluid obtained by extracting the plugs with another 10 cc. of physiological salt solution was added without centrifugation or etherization to the two previous sediments and used for nasal instillation. The etherized specimens were prepared by adding 15 per cent of anesthetic ether, shaking for 10 minutes, storing in the refrigerator overnight, and then centrifuging twice at about 2,000 R P M. The various extracts were inoculated into the same monkey intracerebrally (under local anesthesia), intraperitoneally, and intranasally.

Stools or Enema Returns—When a stool could not be obtained at the end of the period of collection of nasal and oral secretions, the returns from a physiological salt solution enema were used. The amount of solid matter in the enema returns may not be important, since on several occasions we were able to find the virus in enema fluid that was only slightly tinged with brown. When solid fecal material was available it was thoroughly ground in sufficient physiological salt solution, distilled water, or,

¹ We wish to thank Dr Frank Van Schoick of Jackson, Michigan, and Dr Lyman T. Meeks and Dr Benjamin Siebenthal of Indianapolis for their cooperation.

whenever present, in enema fluid to make a 10 or 20 per cent suspension. The preparation of these suspensions for inoculation was the same as that described in a previous publication for the colon contents (1). The methods we used in demonstrating the virus were influenced largely by the results reported by Trask, Paul, and Vignec (3) and by those of Howe and Bodian (6). Paul Trask and Gard (7) observed that they lost very few monkeys when they limited their inoculations of etherized material to the intraperitoneal route and when the amount in a single inoculum did not exceed 20 cc. Using this method for the most part, the total number of positive virus isolations (15 per cent of 53 patients) reported by Trask, Paul, and Vignec (3) was, however, rather low. Howe and Bodian (6), on the other hand, reported the demonstration of virus in 10 of 14 patients (i.e. about 70 per cent) by simple nasal instillation of the untreated stools. We compared the two methods on the first five specimens we obtained (Table I) and found that in this small test, at least, the intraperitoneal

TABLE I
Comparison of Intraperitoneal and Intranasal Methods for Detection of Poliomyelitis Virus in Same Specimen of Stool

Patient	Age	Centrifuged, etherized portion intraperitoneally		Untreated portion intranasally	
		Total dose	Result	Total dose	Result
	yr	"		"	
Wal.	3½	38	P	20	N P
Mc G	2	39	P	18	0
V d B	2	33	0	18	0
Gil	2	40	0	18	0
Kec	12	42	0	18	0

P = paralytic poliomyelitis in inoculated monkey

N P = non paralytic poliomyelitis in inoculated monkey

method was superior to the intranasal although it was evident that nasal instillation of untreated stool can give rise to poliomyelitis infection. We, therefore, combined the two methods with the modification that while a single intraperitoneal inoculum did not exceed 20 cc., each monkey was to receive at least an additional 20 cc. intraperitoneally 24 to 48 hours after the first injection whenever enough material was available.² A portion of the untreated stool (when the specimen was large enough)

² Since submitting this paper for publication we have obtained convincing evidence that in *cynomolgus* monkeys the upper as well as lower parts of the alimentary canal can serve as portals of entry for the virus. Because after nasal instillation of untreated stools or other contaminated specimens most of the material becomes distributed throughout the alimentary tract we now prefer to use *cynomolgus* rather than *rhesus* monkeys, since in the latter only the olfactory mucosa seems to function as a portal of entry for the virus. Using the combined nasal instillation of the untreated portion and intraperitoneal injection of the etherized part of the specimen in *cynomolgus* monkeys three successive enema returns obtained on the 2nd, 4th, and 6th days of paralysis from a 35 year old patient yielded the virus.

or the resuspended centrifuged sediment (after removal of the supernatant liquid for etherization) was given intranasally—1 cc in each nostril daily for 10 days or less if there was not enough material, most monkeys received between 10 and 20 cc in this manner. In a number of monkeys, following the procedure of Howe and Bodian (6), the nasal passages were gently rubbed with a pipe cleaner immediately after the instillation of stool, but one cannot be certain that this increased the incidence of infection by way of the nose. Table II gives a summary of the methods and materials used, and the results obtained. The olfactory bulbs and often also the anterior perforated substance were examined in all monkeys and lesions were regularly absent

TABLE II

Isolation of Poliomyelitis Virus by Various Methods from Different Types of Lower Intestinal Contents

Method of testing for virus	Type of lower intestinal contents		
	Stool	Enema returns	Descending colon contents from fatal cases
One i p	—	0/2	2/2
Two i p	1/4*	1/1	—
One i p + i nas (no p c)	2/5 [0, ?]†	—	—
Two i p + i nas (no p c)	0/1	1/3 [0]	—
One i p + i nas (p c)	—	—	1/1 [+]
Two i p + i nas (p c)	1/3 [?]	3/4 [0, 0, +]	4/4 [+ , + , + , 0]
Total	4/13 (31 per cent)	5/10 (50 per cent)	7/7 (100 per cent)

i p = intraperitoneal, i nas = intranasal, p c = pipe cleaner used after nasal instillation of specimen

*1/4 = virus isolated from one of four specimens

† [0, ?] = data in brackets refer to result of examination of olfactory bulbs of monkeys succumbing with paralysis, in this instance, it means that no lesions were found in the bulbs of one monkey and that in the other they were either not examined or that the sections were unsatisfactory

[0, 0, +] = olfactory bulb lesions absent in 2 monkeys and present in one

when poliomyelitis developed following only intraperitoneal inoculation, the presence of lesions in these sites in some of the monkeys receiving the combined intranasal and intraperitoneal inoculations indicates that in some of the animals the virus invaded the nervous system from the nose by the olfactory pathway. However, the fact that in other paralyzed monkeys which received the combined inoculations, no lesions were present in the olfactory bulbs suggests that in these monkeys infection resulted from the material injected in the peritoneal cavity, and supports the conclusion drawn from the comparative test (Table I) that it is inadvisable to rely on the intranasal method as the sole means of detecting virus.

Saliva and Oral Secretions—Patients, 9 years of age and older, supplied specimens by expectorating into bottles or Petri dishes over a period of 3 days. During this time the secretions were transferred at various intervals to a sterile rubber-stoppered container which was kept in the refrigerator. The total amount collected from

different patients varied between 6 cc. and 75 cc. and in most instances was 20 cc. or more. A portion of the saliva and oral secretions, including the centrifuged sediment, was untreated and instilled intranasally into a monkey while the remainder was etherized and prepared in the same manner as the nasal secretions, and inoculated intracerebrally and intraperitoneally into the same animal.

Urine—Urine was obtained by catheterization from 12 patients 6 of whom had paralysis of the bladder. In only 7 instances however, was it possible to inject the untreated urine into monkeys, in 2 of these 200 cc. was injected intraperitoneally in 4 divided doses (50 cc. in the morning, and 50 cc. in the late afternoon) over a period of 2 days. In 2 others 120 cc. to 150 cc. was given, and in the remaining 3 cases, 80 to 100 cc. was administered in the same manner in 4 divided doses. Because they contained too many bacteria, each of the other specimens was divided into two portions, one of which was filtered through a Berkfeld "V" filter and the other etherized. 100 cc. of each of 3 such specimens were injected intraperitoneally into monkeys. In only 2 instances were smaller amounts (35 cc. and 50 cc. respectively) inoculated.

Animals, Observations and Criteria for Diagnosis of Experimental Poliomyelitis—*Rhesus* monkeys were used and the criteria for positive isolation of poliomyelitis virus were described in the previous communication (1).

RESULTS

The nasal secretions obtained from 22 paralyzed patients during the first 2 weeks of the disease did not yield the virus in a single instance, while in 9 of the same 22 patients the stools or enema returns, collected as previously described, contained readily demonstrable poliomyelitis virus. The nasal secretions of three 5 to 6 year old children with non paralytic poliomyelitis were studied in the same manner with negative results, but tests for virus in the stools of two of these patients were also negative. No virus was found in the saliva and oral secretions of 20 patients, but in only 10 of them were tests made on the lower intestinal contents, and three of these yielded the virus. Tests for virus in large amounts of urine from 12 paralyzed patients were also all negative, but no simultaneous studies were made on the stools of these patients.

Table III contains the data on the tests for virus in the secretions of the 10 paralyzed patients whose lower intestinal contents contained the virus. The results do not support the hypothesis that the virus in the stools has its origin in swallowed nasal secretions and saliva, but are in agreement with our findings on the distribution of virus in fatal cases of human poliomyelitis in which virus was demonstrated in the walls of the pharynx and intestines but not in the nasal mucosa or salivary glands (1). It is not improbable, therefore, that in the valid reports of isolation of virus from nasopharyngeal washings, the virus may have originated from the pharyngeal wall rather than from the nose.

The incidence of 9 positive isolations of virus from the stools of 23 paralytic patients (Table IV) is higher than that (4 out of 38) reported for paralytic cases by Trask, Paul, and Vignec (3) but less than that (10 out of 14) obtained by Howe and Bodian (6). The former investigators (3) were impressed with

TABLE III
Data on Secretions of Paralytic Poliomyelitis Patients with Positive Stools

Patient and sex	Age	Extent of paralysis	Material tested	Specimens collected on		Inoculation				Result
				Day of disease	Day of paralysis	Route	Total dose	No of inoculations	Monkey No	
1 Wal ♀	3½	L	Stool and enema	10	5	nas ip	20 38	10 2	56 57	N P P
			Nasal secretions	7 → 10	2 → 5	ic ip	26 20	2 1	55	O
2 McG ♂	2	rL	Stool	7	5	nas ip	18 39	9 2	58 74	O P (+)
			Nasal secretions	4 → 7	2 → 5	ic ip nas	17 50 80	1 1 4	67	O
3 Fre ♂	5	LU + L	Stool	13	11	nas ip	10 19	5 1	81	P (+)
			Nasal secretions	10 → 13	8 → 11	ic ip nas	3 45 8	2 1 4	88	O
4 Hoy ♀	3	L	Stool	7	4	nas ip	10 17	5 1	82	P (+)
			Nasal secretions	4 → 7	1 → 4	ic ip nas	28 50 80	2 1 4	92	O
5 Cas ♂	2½	L	Stool	5	4	nas ip	20 41	10 2	2 12	P
			Nasal secretions	3 → 5	2 → 4	ic ip nas	2 5 10	1 1 5	2-06	O

6 Joh O	3	L	Exema returns	11	8	lnas f p	18 40	9 2	2-09	P
			Nasal secretions	7 → 10	4 → 7	ic. f p lnas.	2 5 8	1 1 4	2-05	O
7 Jon O	4	U + L	Exema returns	14	8	lnas f p	20 41	10 2	2-13	P
			Nasal secretions	11 → 14	5 → 8	ic. f p lnas	2 6 8	1 1 4	2-10	O
8 Whl O	13	L	Exema returns	6	6	lnas f p	14 47	7 2	39	P (+)
			Nasal secretions	3 → 6	3 → 6	ic. f p	13 95	1 1	21	O
			Saliva and oral secretions	3 → 6	3 → 6	ic. f p	20 210	1 1	26	O
9 Dun O	22	Dysphagia dysphonia	Exema returns	9	8	lnas f p	180 370	9 2	1-18	P (+)
			Nasal secretions	3 → 6	2 → 5	ic. f p lnas	20 80 80	1 1 4	1-07	O
			Saliva and oral secretions	3 → 6	2 → 5	ic. f p lnas	20 60 160	1 1 8	1-66	O
10 Roo O	15	U + L + Intercost and ter minal V IX X XII	Colon contents postmortem	6	4	lnas f p	140 360	7 2	2-01	P
			Saliva and oral secretions	3 → 6	1 → 4	ic. f p lnas	20 70 20	1 1 1	29	O

ic = intracerebral f p = intraperitoneal lnas = intranasal
 U + L = both upper and lower extremities IU = left upper, rL = right lower
 N P = non paralytic poliomyelitis in inoculated monkey
 P = paralytic poliomyelitis in inoculated monkey
 P (+) = paralytic poliomyelitis in inoculated monkey and positive passage

TABLE III
Data on Secretions of Paralytic Poliomyelitis Patients with Positive Stools

Patient and sex	Age	Extent of paralysis	Material tested	Specimens collected on		Inoculation				Result
				Day of disease	Day of paralysis	Route	Total dose	No of inoculations	Monkey No	
1 Wal ♀	3 1/2	L	Stool and enema	10	5	1 nas 1 p	20 38	10 2	56 57	N P P
			Nasal secretions	7 → 10	2 → 5	1 c 1 p	2 6 2 0	2 1	55	O
2 McG ♂	2	rL	Stool	7	5	1 nas 1 p	18 39	9 2	58 74	O P (+)
			Nasal secretions	4 → 7	2 → 5	1 c 1 p 1 nas	1 7 5 0 8 0	1 1 4	67	O
3 Fre ♂	5	IU + L	Stool	13	11	1 nas 1 p	10 19	5 1	81	P (+)
			Nasal secretions	10 → 13	8 → 11	1 c 1 p 1 nas	3 4 5 8	2 1 4	88	O
4 Boy ♀	3	L	Stool	7	4	1 nas 1 p	10 17	5 1	82	P (+)
			Nasal secretions	4 → 7	1 → 4	1 c 1 p 1 nas	2 8 5 0 8 0	2 1 4	92	O
5 Cas ♂	2 1/2	L	Stool	5	4	1 nas 1 p	20 41	10 2	2 12	P
			Nasal secretions	3 → 5	2 → 4	1 c 1 p 1 nas	2 5 10	1 1 5	2-06	O

stools while in the second group only 12 per cent of 60. Statistical analysis shows this to be a most significant difference with a Chi square value of 20.44 and P less than 0.001. One possible explanation of this difference may be that multiplication of the virus is generally more extensive in the younger children. It is of interest in this respect that in contrast to the relative rarity of positive virus isolations from the stools of individuals over 8 years of age, the virus was present in the contents of the descending colon in each of 5 fatal cases in this age group (Table VI). This is a statistically significant difference, and one is naturally led to wonder whether proliferation of the virus is generally more extensive in patients with a fatal termination regardless of age. It is to be recalled, however, that explanations based on unproved assumptions are often wrong, and further investigation of the meaning of these significant differences is indicated.

TABLE VI

Occurrence of Poliovirus in Stools during Life and in Colon Contents Postmortem among Paralytic Patients over 8 Years of Age

Material	No of patients	No positive
Stool or enema returns of living paralytic patients	12	2 (16.7 per cent)
Contents of descending colon postmortem	5	5 (100 per cent)

χ^2 with Yates adjustment for small numbers = 6.97

P is between 0.01 and 0.001

DISCUSSION

In a search for the avenues of elimination of infective virus during the first 2 weeks of paralytic human poliomyelitis, it was found that while the virus could be demonstrated with considerable regularity in the stools (notably in children under 8 years of age), it was not obtained in a single instance from the nasal (not nasopharyngeal) secretions or oral secretions and saliva collected over a period of 3 days or from large amounts of urine. In the light of these findings, the hypothesis that virus is present in the stools because it has been swallowed with nasal or other upper respiratory secretions, becomes untenable, and in view of the demonstration that it is present in the walls of the alimentary tract (1) its origin from these sites is much more probable. It may be suggested, however, that the negative results obtained with the nasal secretions may be due to the presence of an inactivating substance and need not necessarily indicate the absence of virus. If there is such a substance in patients with poliomyelitis it might also be suggested that it should either have been effective in preventing local multiplication in the nasal mucosa, or if it arises in response to infection (and there is no evidence for that assumption) (8) that its presence would thus militate against the spread of infective virus by means of the nasal secretions. However, our studies on the pattern of virus

the fact that with one exception their positive stools were from children 6 years of age or younger. Examination of our own data revealed that while 7 of 11 (64 per cent) fecal specimens were positive in the age group of 5 years and under, only 2 of 12 (17 per cent) were positive in the older patients (one was 13 and the other 22 years old). However, when our data were analyzed statisti-

TABLE IV

Excretion of Virus during the First 2 Weeks of Human Paralytic Poliomyelitis

Material tested	No of specimens (patients)	No positive	Role of age of patient—No positive	
			5 yrs and under	Over 5 yrs
Stool or enema returns	23	9 (39 per cent)	7/11* (64 per cent)	2/12* (17 per cent)
Nasal secretions 3 days	22	0	0/11	0/11
Oral secretions (saliva) 3 days	20	0		0/20
Urine (35–200 cc)	12	0		

* χ^2 with Yates adjustment for small numbers = 3.29

P is between 0.10 and 0.05

TABLE V

Relation between Age of Patient and Incidence of Positive Isolations of Poliomyelitis Virus from Stools

Source of data	Virus isolated from stools of patients	
	under 8 years	8 years and over
Kramer, Hoskwith, and Grossman (5)	2/4 (50 per cent)	1/5 (20 per cent)
Kramer, Gilliam, and Molner (4) (contacts—no clinical "polio")	5/11 (45 per cent)	1/8 (12 per cent)
Howe and Bodian (6)	8/11 (73 per cent)	2/3 (67 per cent)
Trask, Paul, and Vignec (3)	7/21 (33 per cent)	1/32 (3 per cent)
Sabin and Ward	7/11 (64 per cent)	2/12 (17 per cent)
Total	29/58* (50 per cent)	7/60* (12 per cent)

* $\chi^2 = 20.44$, *P* < 0.001

cally³ it was found that Chi square with the Yates adjustment for small numbers was only 3.29 with a value for *P* between 0.10 and 0.05, and, therefore, statistically not significant. But when we combined them with the data which, in our opinion, could be justifiably selected from the literature, a marked difference in the incidence of positive stools was evident when the patients were divided into 2 groups—one under 8 years and the other 8 years and older (Table V). In the first group 50 per cent of 58 individuals yielded positive

³ We are indebted to Mrs. Estelle W. Brown for the statistical analysis of our data.

were combined with those which could justifiably be selected from the literature, the total figures indicating that virus has been isolated from 50 per cent of 58 children under 8 years of age and from 12 per cent of 60 older individuals

5 No support was found for the hypothesis that poliomyelitis virus in the stools originates from swallowed nasal secretions and saliva or oral secretions

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distribution in the human body (1) suggest that the virus neither multiplied in nor invaded the nervous system by way of the nose, because, while it was readily demonstrated in the pharyngeal and intestinal tissues, it was present neither in the nasal mucosa nor in the olfactory bulbs of the cases that were studied

For practical purposes, therefore, it would appear that in human paralytic poliomyelitis the secretions, as they are expelled from the nose or expectorated from the mouth, are probably, as a rule, free from infective virus. However, since the virus has been demonstrated in the pharyngeal tissue postmortem as well as in a certain number of washings obtained by techniques which involved lavage of the pharyngeal wall, it is necessary to determine whether virus that may be present in the pharynx would under natural conditions be swept into the esophagus or might also find its way into the oral secretions and be expelled with them. Since we have recently observed (unpublished data) that in *cynomolgus* monkeys which had developed poliomyelitis after oral feeding of the virus, one can find virus in appreciable amounts in the buccal mucosa and tongue as well as in the pharyngeal wall and washings of the oro-pharyngeal cavity, it would be well to await similar tests on human beings postmortem as well as further studies on oral washings particularly during the first days of the disease in young children, before attempting to form any concept concerning the rôle of the mouth as a site of exit of virus. Although our tests with large amounts of urine were all negative and while the pattern of virus distribution in the human body indicates that there is not sufficient centrifugal progression of virus to permit its invasion of the urinary bladder, it, nevertheless, seems advisable to await actual tests with bladder tissue and further studies on children under 8 years of age before reaching a final decision about the non-infectiousness of urine.

SUMMARY AND CONCLUSIONS

Studies on the elimination of virus in human paralytic poliomyelitis during the first 2 weeks of the disease, revealed the following —

- 1 The nasal (not nasopharyngeal) secretions collected from 22 patients on cotton plugs over a period of 3 days and the saliva and oral secretions expectorated during a similar period by 20 patients failed to yield virus

- 2 In 10 of the patients whose secretions (nasal, oral, or both) were investigated, virus was isolated from single specimens of the lower intestinal contents

- 3 No virus was found in large amounts of urine (up to 200 cc) obtained from 12 patients, 6 of whom had paralysis of the bladder

- 4 In the present tests virus was found 4 times more often in the stools of patients under 8 years of age (64 per cent of 11 cases) than in older individuals (17 per cent of 12 cases). This difference was found to obtain when our data

were combined with those which could justifiably be selected from the literature, the total figures indicating that virus has been isolated from 50 per cent of 58 children under 8 years of age and from 12 per cent of 60 older individuals

5 No support was found for the hypothesis that poliomyelitis virus in the stools originates from swallowed nasal secretions and saliva or oral secretions

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THE INACTIVATING EFFECT OF SULFAPYRIDINE ON THE LEUKOTOXIC ACTION OF BENZENE*

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PLATE 24

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In an attempt to study *in vivo* the rôle of leukocytes in the curative action of sulfonamide drugs for infections, leukopenia was produced in rabbits by the administration of benzene. During the period of intoxication the leukopenic animals were infected with pneumococci and treated with sulfapyridine. Numerous difficulties arose which rendered the experiments indeterminate, and in the course of exploring the difficulties it became apparent that sulfapyridine exerted an inhibitory effect on the toxicity of benzene for the leukopoietic system of rabbits. The data arising from an investigation of the relationship between sulfapyridine and benzene comprise the basis for the present report.

Methods and Materials

The experimental procedure consisted primarily of determining by daily estimations the number of leukocytes in the circulating blood of rabbits receiving sulfapyridine simultaneously with injections of benzene and also in other rabbits receiving benzene alone. The observations have been extended to include additional sets of animals, some of which received sulfapyridine alone others received *p* aminobenzoic acid together with benzene and still others consisted of a normal untreated group.

For comparative purposes, therefore, five groups of rabbits were employed, as follows:

Group 1 Twenty six rabbits received benzene alone. In each experiment two or more animals of this group were included.

Group 2 Twelve rabbits received both sulfapyridine and benzene.

Group 3 Seven rabbits received sulfapyridine alone.

Group 4 Six rabbits received *p* aminobenzoic acid together with injections of benzene.

Group 5 Eleven normal untreated rabbits were also followed.

In addition to observations on the quantitative behavior of the leukopoietic system during the periods of the several treatments, determinations of the urinary excretion of phenols have been made in a limited number of experimental animals. It has long been known that in the animal body benzene is oxidized to form phenols (1) Kracke (2, 3) has made observations on the oxidation of benzene in rabbits and be

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advanced the theory that one or more of the phenolic oxidation products may be responsible for the leukotoxic action rather than the unchanged benzene itself. In the preliminary studies, included in the present report, of a possible chemical explanation of the effect of sulfapyridine on the toxicity of benzene, the excretion of phenols in the urine has been used as an index of the extent of oxidation of benzene to phenolic products.

Adult rabbits weighing about 2.5 kilos were uniformly employed. Leukocyte counts were made each day at the same hour in order that the uncontrolled variables of the white blood cells in the peripheral blood would be reduced to a minimum. Differential counts of stained smears were also made in a limited number of the rabbits. The blood used in making the determinations was derived from the marginal ear veins.

Injections of Benzene—The original procedure of Selling (4) for the dosage and route of injection of benzene was employed. A daily dose of 5.0 cc. of a mixture of equal parts of benzene and olive oil (approximately 1.0 cc. of benzene per kilo of body weight) was injected subcutaneously beneath the skin of the abdomen. A new site was selected for each injection in order to avoid introducing the mixture into an area of edema or inflammation resulting from a prior injection of benzene.

Administration of Sulfapyridine, per Os—Sodium sulfapyridine monohydrate in 5 per cent solution was administered by stomach tube in two equal doses of 1.25 gm. at 9 a.m. and 5 p.m. each day, thus giving a total daily dose of approximately 1.0 gm. per kilo. The benzene was injected at noon, and consequently animals which received both substances were given their first dose of benzene 3 hours after the initial dose of sulfapyridine. Although at the beginning the stomach tube was inadvertently introduced into the trachea, after brief experience the procedure proved simple and the animals evidenced no immediate untoward reaction. No vomiting occurred. The sodium salt was used in order to facilitate the administration of the drug and to insure maximum adsorption.

*Administration of *p*-Aminobenzoic Acid, per Os*—*p*-Aminobenzoic acid was given by stomach tube as a 5 per cent solution of the sodium salt. Two different dosages were used: 0.5 gm. twice daily and 1.0 gm. twice daily.

Determination of Urinary Phenols—The determinations of urinary phenols were done by the method of Folin and Denis (5) using the modified phenol reagent of Folin and Ciocalteu (6, 7). Briefly described, the method consisted of a colorimetric comparison of a filtrate of the urine with a standard solution of phenol after each had been treated with a phosphomolybdic-phosphotungstic reagent. Interfering substances (uric acid, protein) were first removed from the urine by the precipitation with silver lactate and a colloidal iron solution, and the excess silver was precipitated with chloride. The concentration of "free" phenols was obtained from a direct determination on the filtrate, and the concentration of total phenols was found by a similar determination carried out after hydrolysis of the filtrate with hydrochloric acid. The difference between total phenols and "free" phenols represented the conjugated phenol fraction.

EXPERIMENTAL

Group 1 Effects of Benzene Alone—Selling (4) made the observation that, following the daily injection of 2.0 cc. per kilo of the benzene-olive oil mixture,

the number of circulating leukocytes was sharply reduced after a few days, and that the decrease continued progressively until, after continued injections, the animals died with an extreme degree of leukopenia. If the count reached 40 to 720 cells per c. mm., the animals usually died despite the suspension of injections of benzene. Study of the bone marrow revealed that this was the primary seat of the damage rather than the destruction of mature leukocytes in the circulating blood. There was some evidence of destruction in the marrow after 2 days and practically complete aplasia was found after about the ninth injection. The leukopenic effect resulting from intoxication by benzene has been uniformly experienced by many investigators, notably by Kline and Winternitz (8), Weiskotten and his coworkers (9), Camp and Baumgartner (10), Rich and McKee (11), and others.

In the present studies normal rabbits, receiving injections of benzene, have served as controls in each group of experimental animals. The uniformity of the leukopenic effect is attested by the results obtained with 26 rabbits. These rabbits received a daily injection of 5.0 cc. of the mixture of equal parts of benzene and olive oil, and white blood cell counts were made daily. A composite curve derived by averaging the counts obtained in all 26 rabbits is presented in Chart 1. The results indicate the rate of development and severity of the leukopenia. The curve shows an initial rise after the first injection which is in turn followed by a rapid fall during the succeeding 6 days. More specifically, the initial pretreatment average of 8900 rose to 10,200 after 1 day and then fell to 6700 after 2 days, 4700 after 3 days, 3800 after 4 days, 2100 after 5 days, 1500 after 6 days and 880 after 7 days. When the daily injections were continued, death regularly ensued in 12 days or less (average approximately 8 days) and the leukocyte count usually fell below 100.

Although there was some variation in the rapidity with which the leukopenia developed in the individual rabbits, the composite curve gives a reliable picture of the usual trend of the leukocyte count. The count reached a level of 1000 cells per c. mm. or below in from 3 to 9 days in all but one of the animals, and this single exception died on the 5th day with a count of 2200.

Group 2 Effects of Benzene and Sulfapyridine—Twelve animals were injected with benzene according to the regular daily schedule and in addition received sulfapyridine, by oral administration, coincidentally with the benzene. They were maintained on this regime for periods varying from 6 to 19 days. The detailed findings of the individual animals in this group are presented in Table I, the average of results is given in Chart 1. The composite curve of the average of the leukocyte counts of the twelve rabbits (Chart 1) when contrasted with that of the rabbits who received benzene alone reveals the sparing effect of the sulfapyridine on the benzene intoxication. The curve of the group receiving the combined treatment exhibited no sustained downward trend. There were, however, daily fluctuations which were greater than those

seen in normal, untreated rabbits (compare with curve of 11 normal rabbits, Chart 1). A comparison of the initial, pretreatment count with the average counts of the final 3 days of treatment (days 9 to 11 in the chart) reveals that the difference is slight. It will be observed that there is a definite increase in the average counts on the first 3 days after the beginning of treatment, and that the lowest point on the curve, reached on the 8th day, is at 8800 which is only 1500 below the initial count.

TABLE I

Daily Determination of the Number of Leukocytes in the Blood of Rabbits Receiving Sulfapyridine and Benzene Simultaneously

Rabbit No	Days after beginning of treatment												Comment
	Initial	1	2	3	4	5	6	7	8	9	10	11	
4-32	7350	7000	8800	8550	6500	6750	4700	3900	6700	9600	4600	3600	Died on day 11 Continued on benzene and sulfapyridine 8 additional days without leukopenia
4-38	8100	6150	8000	14 300	8950	9150	4000	6200	7200	5050	17 300	10 450	
4-50	5500	6700	8400	6800	7900	13 500	6800	5400					Died on day 8 Sacrificed on day 7 for pathological examination
5-01	13 900	13 800	11 300	13 200	15 500	13 600	15 700	11 700					
5-02	11 900	12 400	14 300	17 600	8900	21 500	18 000	15 200	14 100	13 150	12 950	16 900	Continued 5 more days without leukopenia
5-03	11 650	8900	11 900	15 700	15 900	9600	6700	9500	6700				
5-04	16 500	11 400	9700	11 300	7300	8000	11 600	5300	3000				Died on day 8 Died on day 8
5-05	8500	14 700	11 400	15 400	12 300	9200	17 200	17 800	16 000	15 050	9100	12 500	
5-06	11 400	17 700	12 800	12 400	10 600	9200	9400	7900	10 900	5650	8200	12 400	Continued 7 more days Moderate leukopenia on last day
2-37	11 800	10 500	14 200	10 500	7600	11 600	7400	9100	5000	3800	6600	6700	
2-05	14 000	18 200	19 800	12 800	13 200	15 100	11 300	10 500	10 300	22 000	14 400	13 300	Died on day 5
2-07	3800	10 500	8900	12 300	11 800								

The detailed data, recorded in Table I, give the daily counts of these rabbits for the first 12 days. Only 6 of the 123 counts recorded fall below 5000 cells per c mm. The lowest leukocyte count in any of the animals was 3000 (rabbit 5-04) occurring on the day of death, which was 8 days after the beginning of benzene and sulfapyridine administration. One other animal of this group (rabbit 4-32) died with a moderate leukopenia, 3600 cells per c mm, after 11 days. The count of 5400 occurring in rabbit 4-50 at the time of death on the 8th day was the same as the initial count of 5500.

Differential leukocyte counts were made at 3 to 4 day intervals on seven of the animals of this group. In general, the variations observed were not greater

than those that occur in normal animals. The percentage of polymorphonuclear leukocytes increased moderately in three of the animals, decreased in two, and remained essentially unchanged in two. Immature cells of the granulocytic series, other than metamyelocytes, were not found on the smears.

Concerning the inactivating effect of sulfapyridine on the leukotoxic action of benzene, it is interesting to note that other toxic manifestations of benzene poisoning did not appear to be entirely eliminated. Anorexia, loss of weight, extreme weakness, and diarrhea occurred in varying degrees of severity in many animals of the control group and also members of the sulfapyridine and benzene group which maintained normal counts. Even death occurred in four of the animals receiving benzene and sulfapyridine (rabbits 4-32, 4-50, 5-03, 2-07) although the leukocyte count was normal in two and the leukopenia was not unusually severe in the other two. However, since only four of the animals of this group died while under observation whereas all of the animals receiving continued injections of benzene alone died within 12 days, it appears likely that sulfapyridine served as an antidote for the general intoxication but to a considerably less degree than for the leukotoxic effect of benzene.

Attempts were made to determine how long the beginning of sulfapyridine therapy might be delayed after starting benzene injections and still obtain the antileukopenic effect. In four animals sulfapyridine was first given only after the leukocyte count had been reduced to about 3000 cells per c. mm. In three of the rabbits definite inhibition of the continued development of leukopenia was indicated by the fact that subsequent counts either failed to decrease progressively or actually increased. In two animals which were started on sulfapyridine when the count had reached 1000 per c. mm., the leukopenia followed an unmodified course and death occurred on the 2nd day after beginning sulfapyridine. Although these experiments are not sufficiently complete to justify final conclusions, they suggest that the effectiveness of sulfapyridine in counteracting the toxicity of benzene is determined by the extent of the damage produced by the benzene prior to the beginning of sulfapyridine therapy.

Group 3 Effects of Sulfapyridine Alone—In a third group of animals sulfapyridine was given without benzene. Seven rabbits received 1.25 gm. *per os* twice daily for periods of 11 to 25 days to determine the effect of the drug on the number of circulating leukocytes. Interest was directed toward the possibility that sulfapyridine might effect stimulation of the marrow as reflected by leukocytosis which might in itself antagonize the action of benzene. That such a possibility warrants consideration is indicated by the studies of Weiskotten and Steensland (12) who reported the failure of benzene to produce leukopenia in four rabbits suffering from spontaneous acute infections. The investigators observed that three of the four rabbits developed leukocytosis and in all of them the percentage of polymorphonuclear leukocytes increased

under benzene treatment The failure of benzene to produce leukopenia under these conditions suggested to the authors that the marrow of rabbits, under the stimulating leukopoietic effect of infection, is able to withstand the toxicity of benzene or is more resistant to its action

A curve of the average leukocyte count of the seven animals receiving sulfapyridine alone is included in Chart 1 As in the case of the animals receiving sulfapyridine and benzene, the daily fluctuations of the count in this group of animals were greater than those observed in normal animals However, in the composite average plotted in the chart the counts of a single animal were responsible for a large part of the wide variations This animal is the only one of the seven in which the response of the leukocyte count gave evidence of a stimulating effect, and even in this instance, the count fluctuated irregularly between 12,000 and 31,600 The leukocyte count of the remaining six animals tended to show a downward trend which was most striking in one animal in which a count of 3800 was reached after 9 days and in another in which the count was 2600 after 16 days Differential leukocyte counts in this group of rabbits revealed no increase in the percentage of granulocytes during the period of administration of sulfapyridine

Because of the relatively wide fluctuations observed in the group of rabbits receiving sulfapyridine alone, as well as in those receiving sulfapyridine plus benzene, it seems not unlikely that sulfapyridine exerts an effect on the leukopoietic system The nature of the reaction has not been studied However, the findings do not warrant the explanation of the antileukopenic action of sulfapyridine as being referable to an overcompensating leukocytosis

Group 4 Effects of Benzene and p-Aminobenzoic Acid—It has been demonstrated by Woods (13) that *p*-aminobenzoic acid inhibits the bacteriostatic effect *in vitro* of sulfanilamide Selbie (14) found that it also nullified the action of sulfanilamide in experimental streptococcal infections in mice, and our results (15) indicated a similar inhibitory effect on the action of sulfapyridine in experimental pneumococcal infections of mice Woods (13) advanced the theory that *p*-aminobenzoic acid is essential for the growth of the organism and that the sulfonamide drugs interfere with its utilization by virtue of their structural relationship to *p*-aminobenzoic acid

In view of the similarity in chemical structure between *p*-aminobenzoic acid and sulfapyridine and the antagonistic relationship between the two drugs in the bacterial studies, six animals were given *p*-aminobenzoic acid coincidentally with benzene as an additional control on the relative specificity of sulfapyridine Three of them received 1.0 gm. twice daily and three 0.5 gm. twice daily Mol for mol, the 1.0 gm. dose was larger than the dose of sulfapyridine used and the 0.5 gm. dose was smaller The fall in the leukocyte count of the six rabbits was in no way different from that observed in rabbits receiving benzene alone The curve of the average counts given in Chart 1 reveals how closely the response of this group paralleled that of the group receiving benzene alone

An attempt was made to determine whether *p* aminobenzoic acid, in accordance with its inhibitory effect on the antibacterial action of sulfapyridine, would influence the effect of sulfapyridine on the response of the leukocytes to the injection of benzene. For this purpose three rabbits received sulfapyridine plus *p*-aminobenzoic acid plus benzene. The experiment failed of conclusive results, because all three animals which received the three substances simultaneously became ill very rapidly and died within 3 to 5 days. The observations of James (16) on the depletion of acetate precursors as a cause of toxicity from sulfanilamide and sulfapyridine may be pertinent to the acute toxicity displayed by these animals.

Group 5 Normal Rabbits—Although there are many reports in the literature on the course of the leukocyte counts of normal rabbits, daily estimations of the white blood cells were made on eleven normal rabbits as an additional control in the present study. When these rabbits are considered as a group and attention is directed to the average of the leukocyte counts of the 11 animals (Chart 1), it will be observed that the daily variations are small. The range between the highest and lowest average counts is less than 1000 cells, and therefore the average counts during the period of observation did not show variations of more than 9 per cent.

Chart 1—In Chart 1 all of the results which have been separately described are consolidated. The salient features are as follows:

- 1 The rapid, progressive fall of the leukocyte count in the group receiving benzene alone and the group receiving benzene plus *p* aminobenzoic acid.

- 2 The absence of a significant fall in the curve of the average leukocyte count of the group receiving benzene plus sulfapyridine.

- 3 The lack of evidence of sustained leukocytosis in the curve of the animals receiving sulfapyridine alone.

- 4 The wide daily fluctuations in the average leukocyte count of the group receiving sulfapyridine plus benzene as well as the group receiving sulfapyridine alone when compared with those of a group of normal rabbits.

Chart 2—In Chart 2 are recorded the leukocyte counts of one animal of the benzene group and one animal of the sulfapyridine plus benzene group which were sacrificed for pathological examination on the day of the eighth injection. The chart makes evident the rapidly developing leukopenia of the rabbit on benzene alone and the maintenance of normal counts, ranging between 11,300 and 15,700, in the rabbit receiving benzene plus sulfapyridine. Photomicrographs of the femoral marrow of the two rabbits, along with the marrow of a normal untreated rabbit are presented in Figs. 1 to 3. The sections of marrow are from corresponding areas, and in each case are representative of the condition of the marrow in all areas sampled. They serve primarily to illustrate the influence of sulfapyridine in preventing the extreme aplasia which consistently results from injections of benzene in rabbits. The marrow of the animal which received sulfapyridine concurrently with the benzene shows an

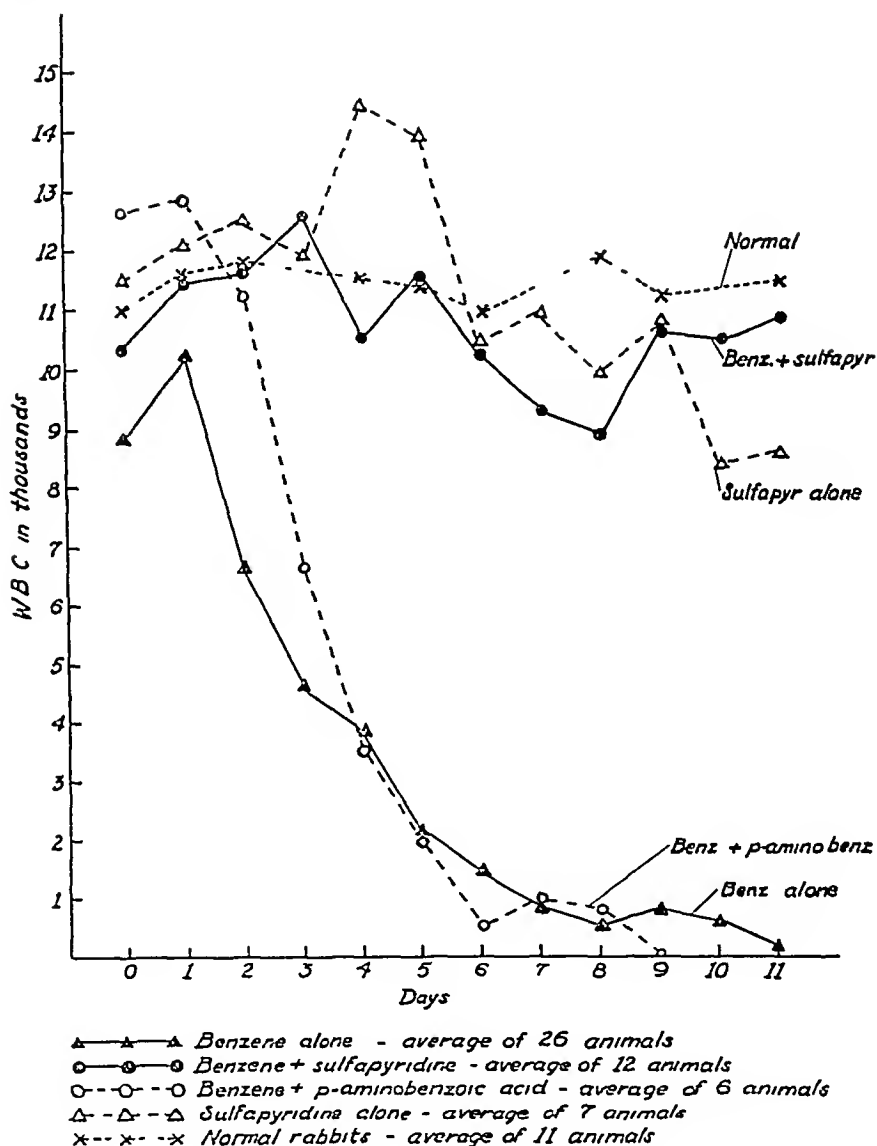


CHART 1 Curves of the composite average of the daily leukocyte counts of all the rabbits in each group

abundance of hemopoietic tissue Furthermore, some of the megakaryocytes have hyperchromic and pyknotic nuclei The latter finding differs from the usual appearance of the marrow often found in untreated rabbits In spite of

the wide variations which may exist in normal rabbits, the morphological evidence of "irritation" or "stimulation" suggests the special effect induced by sulfapyridine alone or in combination with some of the products of benzene. A detailed analysis of the reaction of hemopoietic tissue to the chemical reagents requires qualitative studies not contained in the scope of this report. How

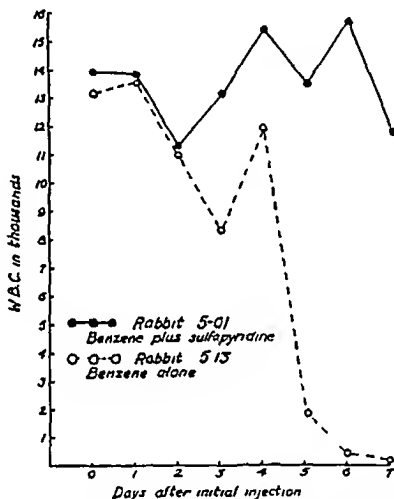


CHART 2 Daily leukocyte counts of rabbit 5-01 (benzene and sulfapyridine) and rabbit 5-13 (benzene alone) which were sacrificed for pathological examination. See Figs. 1 and 2.

ever, the comparable sections of bone marrow from the experimental animals reflect the quantitative results noted in the leukocytes of the circulating blood.

Urinary Excretion of Phenols—As stated earlier, benzene injected into the animal body has been reported to be oxidized in part to phenols, (1). That one or more of the phenolic oxidation products may be responsible for the leukotoxic effect of the injected benzene has been suggested, (2, 3). In accordance with this possibility, the urinary phenols were studied in a small group of animals to determine whether the administration of sulfapyridine quantitatively influenced the excretion of phenols following subcutaneous in

jections of benzene The animals were kept constantly in metabolism cages which allowed the collection of 24 hour urine specimens, and benzene and sulfapyridine were administered as in the preceding experiments

In the course of a series of studies on benzene leukopenia, Brewer and Weiskotten (17) made estimations of the urinary phenols of two rabbits They observed fivefold increases in the output of phenols after injection of a benzene-olive oil mixture Our results in three rabbits receiving benzene alone are in accord with their findings Normally, the untreated rabbits excreted 90 to 140 mg of phenols in 24 hours The excretion doubled, however, in the 24 hour period after the first injection of benzene and continued to increase after subsequent injections One animal excreted 590 mg of phenols in the second 24 hours, and the other two excreted maximum amounts of 575 mg and 658 mg on the 4th day

When sulfapyridine was given to three animals in conjunction with benzene, the excretion of urinary phenols differed from that described in the animals receiving benzene alone In each of the three the increase in output of total phenols was substantially less than in the rabbits receiving benzene alone Furthermore, in two of this group there was in addition a decided increase in the percentage of conjugated phenols For example, one animal with an excretion of 75 to 136 mg of total phenols in 24 hours under normal conditions, showed no increase in the first 2 days after beginning the administration of benzene and sulfapyridine Furthermore, the 24 hour values for total phenols did not exceed 200 mg during 5 days of continued treatment Another animal showed no increased excretion in the first 24 hours When, however, in the second 24 hours the total phenols were doubled, 50 per cent of the total was present in the conjugated form

The record of a single animal in which a study of the urinary phenols was made during periods of the administration of benzene alone, sulfapyridine alone, and benzene plus sulfapyridine, serves to illustrate the results obtained Chart 3 presents in graphic form the data on the excretion of urinary phenols of this rabbit

It will be noted that sulfapyridine alone when given over a 3 day period exerted no appreciable effect on the phenol excretion Daily injections of benzene caused a marked increase in the urinary phenols and a maximum of 575 mg was attained in the 24 hours following the fourth injection In contrast with the results using benzene alone, during a 3 day period in which sulfapyridine was administered coincidentally with benzene, the increase in total phenols was slight and there was a definite relative increase in the fraction of conjugated phenols The delayed rise which occurred on the second day after discontinuing treatment may be interpreted as being dependent upon the slow absorption of the benzene-oil mixture

Although the number of observations on the urinary excretion of phenols is too few to justify detailed conclusions the differences in the phenolic output of rabbits receiving sulfapyridine plus benzene as contrasted with the animals injected with benzene alone have been constant. Concerning the mechanism of the effect of sulfapyridine on the phenol excretion, several possibilities warrant consideration. On the assumption that the limited increase in the excretion of phenols, when benzene was injected into rabbits receiving sulfapyridine, reflects an actual decrease in the formation of phenols from benzene,

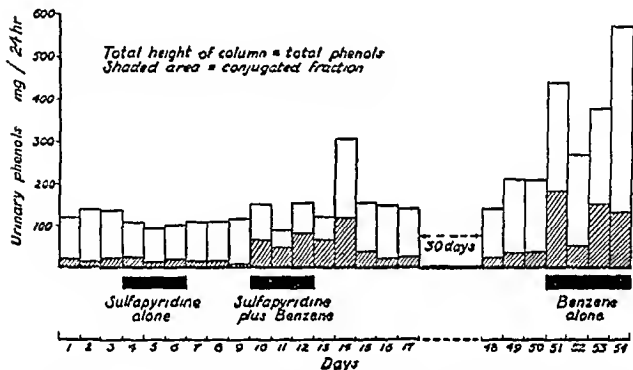


CHART 3 Urinary excretion of total and conjugated phenols of rabbit 212 during periods of treatment with sulfapyridine alone, sulfapyridine plus benzene, and benzene alone

it seems possible that the presence of sulfapyridine in the tissues may influence the oxidation of benzene. Concerning the possible effect of sulfapyridine through the oxidative processes it may also be noted that benzene may be oxidized in the body to end products other than phenols. Jaffe (18) has reported that disruption of the benzene ring resulting in the formation of the straight chained muconic acid occurs in addition to the oxidation to phenols. On theoretical grounds, therefore, sulfapyridine may favor the processes involved in the breakdown of the ring and thereby reduce the amount of phenols or increase the formation of other, as yet undefined end products. Further study is required to appraise the validity of these possibilities. However, since the introduction of sulfapyridine is associated with the inhibition of the development of leukopenia and also brings about a reduction in the excretion of

free phenols which follows injections of benzene, the findings lend support to the possibility that phenolic substances or other end-products may be responsible for the leukotoxic action of benzene

SUMMARY

1 In rabbits receiving subcutaneous injections of benzene, the simultaneous administration of sulfapyridine, *per os*, prevented the development of leukopenia. The sparing effect of sulfapyridine on the intoxication of the leukopoietic tissue by benzene was demonstrated not only by the range of daily leukocyte counts but also by microscopic examination of the bone marrow of treated animals.

2 The administration of para-aminobenzoic acid failed to inhibit the leukotoxic action of benzene.

3 The administration of sulfapyridine alone to rabbits was followed by daily variation in the number of leukocytes in the circulating blood but persistent leukocytosis was not observed. The inhibiting action of sulfapyridine was not found to be referable to the development of an overcompensating leukocytosis.

4 In preliminary experiments, the excretion of phenols by rabbits receiving sulfapyridine together with benzene differed from that observed in animals receiving benzene alone. In the former group, the rise in the excretion of total phenols was not so high as in the latter group, but the percentage of combined phenols was greater. The possible significance of the findings has been discussed.

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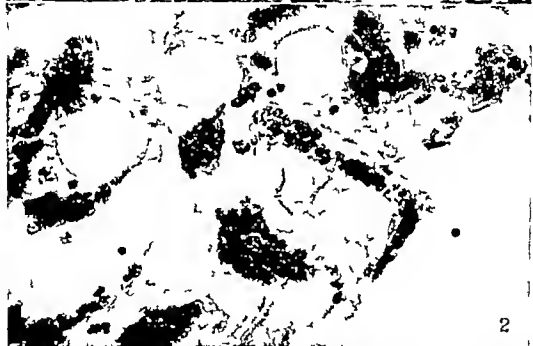
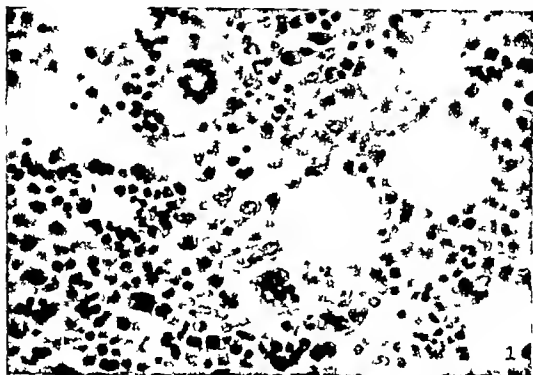
EXPLANATION OF PLATE 24

We are indebted to Dr Irving Graef, Department of Pathology, New York University College of Medicine, for the description of the sections appearing in Figs 1 to 3

FIG 1 *Rabbit 5-01* (combined benzene and sulfapyridine) Sections of the femur marrow (sections of rib and vertebra are similar) reveal fatty marrow with large islands of hemopoietic tissue, normal types of cells and many young eosinophilic leukocytes. No "toxic" or degenerative forms were observed. Some megacaryocytes reveal hyperchromic and pyknotic nuclei but many are quite normal.

FIG 2 *Rabbit 5-13* (benzene) Sections of the femur marrow (rib and vertebra are identical) reveal complete depletion of hemopoietic cells, congested and dilated marrow sinusoids and large fatty lobules. The nuclei of the fat cells have disappeared, as well as the hemopoietic cells.

FIG 3 *Normal Rabbit* Sections of the femur marrow (illustrated by the accompanying photograph) reveal a mixture of fatty tissue, islands of hemopoietic cells and marrow sinusoids. The pattern is fairly uniform, the cells are distributed in moderate numbers and exhibit the usual normal types.



A QUANTITATIVE STUDY OF THE SCARLET FEVER TOXIN-ANTITOXIN FLOCCULATION REACTION*

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Since the discovery of scarlet fever toxin (Dick and Dick (1), Dochez (2)) there have been numerous attempts to isolate the active substance from the culture filtrates of scarlatinal strains of hemolytic streptococcus. Although the erythrogenic toxin has been purified to varying degrees, there has been little agreement in the literature as to its chemical nature or regarding the extent of purification attained. Some of the earlier workers suggested that the toxin might be a nitrogenous carbohydrate (Korschun *et al.* (3), Stock (4)). Dick and Boor (5) isolated a fraction of reduced nitrogen content containing 30,000 skin test doses per mg. The most active preparation of scarlet fever toxin that has been reported was that isolated by Stock (6). His purest material contained 11 per cent nitrogen and 10,000,000 skin test doses per mg. Stock's purest preparations presumably consist mainly of protein, but little has been stated regarding their chemical properties. On the other hand Barron, Dick, and Lyman (7) have recently carried out an extensive chemical study on material containing only 30,000 s.t.d.¹ per mg., which from electrophoresis data they regard as about 35 per cent pure. The latter authors conclude that scarlet fever toxin is a protein of low molecular weight and unusual stability. Finally, Koerber and Bunney (8) have described a "protein free" scarlet fever toxin which they isolated from a casein hydrolysate medium. Their preparations contain only 0.7 to 1.0 per cent nitrogen and about 100,000 s.t.d. per mg. For further references to work on the purification of scarlet fever toxin the paper by Stock (6) should be consulted.

In view of the contradictory results obtained by other workers, we have deemed it important to find some independent method for estimating the activity of pure scarlet fever toxin before actually attempting to isolate it in its purest form. The flocculation reaction appeared to offer possibilities for such a method. In a quantitative study of the Ramon diphtheria flocculation reaction, Pappenheimer and Robinson (9) showed that, within the equivalence zone, the nitrogen precipitated from a given amount of antitoxin varied linearly with the toxin added. The slope of this straight line was shown to represent

* The expenses of this work have been defrayed by a generous grant from the Commonwealth Fund.

¹ The abbreviation s.t.d. for skin test doses will be used throughout the rest of this paper.

the nitrogen of pure diphtheria toxin per Lf unit. Moreover, the method was found independent of the purity of the toxin and antitoxin preparations employed. Rane and Wyman (10) have described a flocculation reaction between scarlet fever toxin and antitoxin which they regard as a specific antigen-antibody reaction because of the close *in vivo-in vitro* correlation which they obtained using a great many preparations of toxin and antitoxin. If this flocculation reaction is indeed specific, then it should be possible to estimate the nitrogen per flocculating unit of pure scarlet fever toxin from a quantitative study of its reaction with antitoxin, providing that the reaction is analogous to the diphtheria system. Unfortunately, Evans and Gottschall (11) and Bunney and Koerber (12) have failed to obtain satisfactory correlation between the flocculation reaction and *in vivo* titrations of scarlet fever toxin, and the latter workers have expressed some doubt regarding the specificity of the reaction. It is hardly necessary to point out that before the flocculation reaction can be used to estimate the activity of pure scarlet fever toxin, its specificity must be established beyond reasonable doubt. With these factors in mind we have undertaken a quantitative study of the flocculation reaction of Rane and Wyman (10) between scarlet fever toxin and antitoxin.

EXPERIMENTAL

Cultures—Toxins were prepared using two different scarlatinal strains of hemolytic streptococcus. Strain NY No. 5 has been most commonly used commercially for the production of scarlet fever toxin. It has been shown by Rane and Wyman (13) that strain No. 594 produces several times as much toxin as NY No. 5. Evidence that the toxin produced by No. 594 is identical with that of NY No. 5 has been given by Rane and Wyman, and by Plummer and Fraser (14). We are indebted to Dr. Leo Rane of the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts, for cultures of the two strains.

Preparation of Toxin—60 liters of culture of strain No. 594 and 30 liters of NY No. 5 were grown in 5 gal. pyrex carboys on the casein hydrolysate medium described elsewhere (15). After 60 hours growth at 35°C. with neutralization and continuous stirring the cultures were centrifuged and the supernate sterilized by Seitz filtration. The average yield of erythrogenic toxin from No. 594 was 9 Lf/cc (ca. 300,000 S.T.D. per cc), and from NY No. 5 was 2 Lf/cc (ca. 60,000 S.T.D. per cc).

Concentration and Partial Purification of Toxin—The toxic filtrates from the two strains were concentrated and purified separately by the same procedure as follows. The culture filtrate was concentrated *in vacuo* at 30–35°C. to about 1/7 its original volume. The active material was then precipitated by adding 500 gm. of ammonium sulfate for each liter of concentrate and enough ammonia to keep the solution between pH 6.5 and 7.0. On standing overnight at room temperature, the toxin formed a dark flocculent layer near the surface. A large amount of crystalline sediment also appeared. The precipitates were filtered by suction and dissolved by dialysis against running water. After thorough dialysis the solution remained turbid and contained some precipitate which was removed by filtration through hard paper and discarded.

The volume at this point was 2 to 5 per cent that of the original culture filtrate. Ammonium sulfate was added to four tenths saturation and a small amount of dark brown inactive precipitate was removed by filtration. This filtrate was brought to 0.75 saturation with ammonium sulfate and the toxin precipitated. The dark brown precipitate containing the toxin was separated by filtration through paper and dissolved in a small volume of water. The solution was dialysed against changes of distilled water and finally against Sprenson's phosphate buffer at pH 6.8 containing 1:5000 merthiolate. The flocculation reaction was used at each step to follow the fractionation of the toxin. No attempt was made to effect further purification.

Properties of Partially Purified Toxin—From 60 liters of No. 594 culture filtrate, 400 cc. of purified toxin containing 950 Lf/cc. 3×10^7 s.t.d./cc., and 0.895 mg. nitrogen per cc. were obtained at this point, representing an overall yield of about 70 per cent. From 30 liters of NY 533 cc. of purified toxin

TABLE I
Some Properties of Partially Purified Scarlet Fever Toxin

	Strain No. 594	Strain NY 5
Nitrogen content (per cent)	14.0	12.5
Activity (skin test doses per mg.)		
Rabbit	8,000,000	12,000,000
Human	8,000,000	12,000,000
Mg. nitrogen per Lf unit	0.00054	0.00035
Flocculation time for 38 Lf per cc. at 48°C. using antitoxin 531B (min.)	30	30
Per cent precipitable*	43	66

* Calculated using 0.00023 mg. nitrogen per Lf unit of toxin and based on the total nitrogen in the toxin.

were obtained, containing 800 Lf/cc., 2×10^7 s.t.d./cc., and 0.345 mg. nitrogen per cc., a yield of about 45 per cent. Both preparations gave strongly positive biuret, Millon and xanthoproteic tests in 0.3 per cent solution. Both preparations contained some material which was coagulated by heating to 100°C. Dilutions of the 594 toxin were injected intravenously into a few rabbits. These must be regarded as only preliminary experiments but have indicated that the M.L.D. for 2 to 3 kilo Chinchilla rabbits is about 50 Lf (1,500,000 s.t.d.) by the intravenous route. One rabbit given 95 Lf and 300 units of antitoxin survived. Rane and Wyman (13) report 180 Lf lethal for 5 lb Chinchilla rabbits when injected subcutaneously. Some properties of the partially purified toxin after further dialysis are summarized in Table I.

In agreement with other workers (Kodama (16), Hooker and Follensby (17), and Barron *et al.* (7)) we have found the toxin to be relatively resistant to the action of the proteolytic enzymes, pepsin, papain and trypsin. The flocculating power of the toxin was *not* impaired by treatment with these enzymes.

That these toxin solutions contained other streptococcal proteins was shown by a positive ring test when a 1/100 dilution of the concentrated toxin was added to an immune rabbit serum prepared against a smooth strain of hemolytic streptococcus

*Antitoxins*²—Antitoxin No 2101 (Squibb Laboratories) was whole horse serum Antitoxin No 24739, also obtained from Squibb Laboratories, was a commercial preparation of pseudoglobulin Antitoxins No 531A and 531B, obtained from the National Drug Company, were from different bleedings from the same horse They were solutions of the water-soluble globulin precipitated between 0.3 and 0.5 saturation with ammonium sulfate Antitoxin No 47E (National Drug Company) was a sample of enzyme-digested antitoxic pseudoglobulin Antitoxin No S113A was a pseudoglobulin preparation received from the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts The flocculation times of the various antitoxins varied between 25 and 130 minutes at 48°C with 38 Lf units of toxin (See Table III)

The Flocculation Reaction—The flocculation tests were done in the usual manner at 48°C with a constant amount of toxin and increasing amounts of antitoxin In evaluating flocculation titers or Lf units per cc we have used the arbitrary standard set up by Rane and Wyman (10) In the quantitative studies, the amount of antitoxin in each tube was kept constant at 600 units Increasing amounts of toxin were added and the volume in each tube was adjusted to 5 cc with saline These mixtures were set in a water bath at 38°C for 3 hours and overnight in the cold The tubes were then centrifuged, the supernates drawn off for test, and the precipitates broken up and washed three times with chilled saline Nitrogen in the precipitates was determined by the micro-Kjeldahl method The supernates were tested for excess toxin by skin tests in rabbits All tests were carried out in duplicate

Skin Tests—The skin reactivity of toxin solutions and the supernates from the flocculation tests was determined by injecting graded dilutions into the skin of white or grey Chinchilla rabbits The dilutions were made in saline in such a manner that each successive tube in a series contained half as much toxin as the preceding tube The reactions after 24 hours and 40 hours were compared with similar reactions produced in the same rabbits by the Standard National Institute of Health toxin³ Three rabbits were used for each series of titrations and the results averaged In the discussion of our results we have regarded the skin test values as significant only within ± 50 per cent

The skin reactivity of the concentrated and purified toxin preparations was confirmed *exactly* by tests carried out on susceptible human subjects using the Standard National Institute of Health toxin for comparison We are greatly indebted to Dr George F Leonard of the Biological Laboratories, E R Squibb and Sons, for carrying out the human skin tests

² We are greatly indebted to Dr W E Bunney of the Squibb Laboratories, New Brunswick, New Jersey, to Mr A M Slee of the National Drug Company, Swiftwater, Pennsylvania, and to Dr E S Robinson of the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts, for the antitoxins used in this work

³ This toxin was supplied by Dr M V Veldee of the National Institute of Health It was labeled 30,000 s r d per cc

TABLE II

Quantitative Flocculation of Antitoxic Pseudoglobulin 531

Increasing amounts of scarlet fever toxin added to 600 units of antitoxin

I	II	III	IV	V	VI	VII	VIII
Toxin added	Toxin nitrogen	Nitrogen in precipitate	Antitoxin nitrogen in precipitate III + II	Ratio, A nitrogen T nitrogen IV + II	Total skin test doses added	Total skin test doses§ in supernate	Strain No
Lf	mg	mg					
300	0.069	0.174			9 000 000	5 000 000	594
340	0.078	0.400			10 000 000	2 000 000	NY5
350	0.081	0.502			10 500 000		594
400	0.092	0.616			12 000 000		594
440¶	0.100	0.625			13 000 000	1,000 000	NY5
450¶	0.103	0.655			13 500 000	550 000	594
500	0.115	0.675	0.560	4.9	15,000 000		594
540	0.124	0.680	0.556	4.5	16 000,000	150 000	NY5
600	0.138	0.700	0.562	4.1	18 000 000	150 000	594
640	0.147	0.705	0.558	3.8	19 000 000	100 000	NY5
700	0.161	0.719	0.558	3.5	21 000 000		594
740¶	0.164	0.715			22 000 000	100 000	NY5
800¶	0.184	0.710			24,000 000	3 000 000	594
840	0.193	0.666			25 000 000		NY5
900	0.207	0.513			27 000 000		594
940	0.216	0.162			28 000 000	15 000 000	NY5
1000	0.230	0.224			30 000 000		594
600	0.138	—	anti Ea**	—	18 000 000	7 500 000††	594
600	0.138	1.340	Ea anti Ea	—	18 000 000	5 000 000††	594
—	—	1.360	Ea anti Ea	—	—	none	594

* The nitrogen precipitated by 500 Lf units of toxin (column III) subtracted from that precipitated by 700 Lf and divided by 200 gives 0.00072 mg. nitrogen per Lf unit of toxin. The figure used in column II, however, is 0.00023 mg. of nitrogen per Lf unit, the average obtained from three titrations including the above.

† All values except the one marked || are the averages of duplicate determinations.

‡ Values are calculated only for points of complete precipitation in the equivalence zone.

§ These values were determined in the skin of rabbits. They represent averages of tests on three rabbits.

¶ Incomplete flocculation.

** Ea = egg albumin.

†† These two values are undoubtedly low. They are included because they were determined on the same rabbits and show that there is no significant amount of toxin adsorbed to the egg albumin anti egg albumin precipitate.

RESULTS

The results of titrations of antitoxin 531 with both NY5 and 594 scarlet fever toxins are given in detail in Table II and plotted in Fig. 1. A quantitative diphtheria toxin antitoxin titration is also plotted in Fig. 1 for comparison.

The reaction between scarlatinal toxin and antitoxin resembles the quantitative diphtheria toxin-antitoxin reaction in several respects. Thus, for example, precipitation occurs within a narrow zone only. Moreover, the Danysz phenomenon is readily demonstrable by flocculation in the inhibition zone of antitoxin excess and although not indicated in Table II, its magnitude is the same as with the diphtheria system. Thus after 200 Lf of scarlet fever toxin were incubated with 600 units of antitoxin, no flocculation occurred. However,

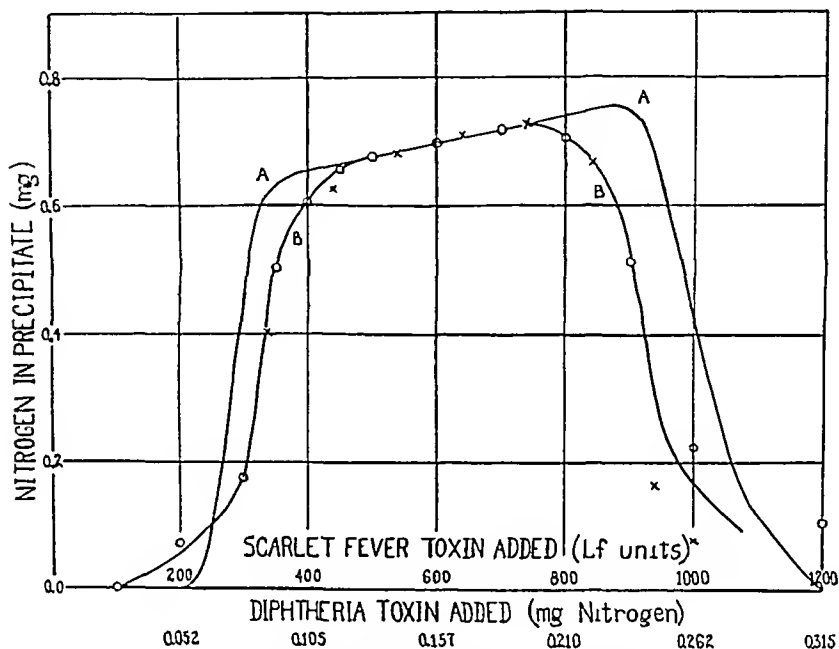


FIG 1 Quantitative toxin-antitoxin flocculation reaction. Curve A, diphtheria system, Pappenheimer and Robinson (9). Curve B, scarlet fever system. O = 594 toxin. x = NY5 toxin.

when the mixture was then treated with increasing amounts of toxin, only 200 units of antitoxin were found by flocculation rather than 400 units expected if there were no Danysz phenomenon. In spite of these similarities, certain important quantitative differences exist between the diphtheria and scarlet fever toxin-antitoxin reactions. Although the width of the flocculation zone is almost exactly the same for both reactions, the region of nearly complete precipitation is more than twice as broad for the diphtheria toxin-antitoxin system as for the scarlet fever toxin-antitoxin system. This is not only true for antitoxin No. 531 shown in Fig. 1, but holds for all the scarlet fever antitoxins which we have studied. In fact antitoxin No. 531 was chosen for

detailed study because its zone of nearly complete precipitation was the broadest of all the scarlet fever antitoxins we have examined.

Several quantitative titrations with various antitoxin preparations are summarized in Table III.

TABLE III
Results from Various Toxin-Antitoxin Titrations

I	II	III	IV	V	VI	VII	VIII
Antoxin No.	Units antitoxin per cc. by flocculation	Flocculation time (38 Lf/cc at 48°C)	Toxin strain No.	Toxin nitrogen per Lf	Maximum specifically precipitable antitoxin nitrogen from 600 units antitoxin	Total nit. gen. specifically precipitated	Ratio $\frac{A \text{ nitrogen}}{T \text{ nitrogen}}$ at flocculation point
		min			mg	per cent	
2101	133	25	594	(0.00036)†	0.581	1.0	4.2
531A	315	35	594	0.00022	0.560	1.9	4.1
531B	250	30	NY5	0.00021	0.552	2.0	4.0
S113A	940	130	594	—†	0.612	3.9	4.4
24739	880	110	594	0.00024	0.484	2.4	3.5
47E	720	46	594	—†	0.458	1.9	3.3

* Calculated using 0.00023 mg. nitrogen per Lf unit of toxin.

† The results of this titration were irregularly high, indicating that the precipitates were not washed sufficiently.

‡ The equivalence zone was too narrow to calculate a value.

DISCUSSION

In the previous study of the diphtheria flocculation reaction (9) it was assumed that throughout the equivalence zone precipitation was essentially complete, and that even if the specific toxin-antitoxin complex were slightly soluble within the zone, no appreciable dissociation occurred, since no toxin could be detected in the supernate by the sensitive rabbit intracutaneous test. This is not true in the present instance. Even at the flocculation point the scarlet fever toxin-antitoxin complex is somewhat soluble and the soluble complex is apparently dissociated to an appreciable extent. Since it is practically completely dissociated at high dilutions, the skin test method may be used to determine roughly its solubility. Column III of Table IV shows that the solubility of the toxin-antitoxin complex at the flocculation point remains practically constant over a 50-fold range of concentration. The solubility at ice box temperature corresponds to about 1 Lf per cc. (30,000 S.T.D. per cc.).

It is well established that antigen-antibody reactions are reversible and that under certain conditions the antigen-antibody complex may dissociate. Glenny, Pope, and Waddington (18) have presented evidence that the diphtheria toxin-antitoxin complex may dissociate to an appreciable extent upon dilution, particularly when antitoxins of low "avidity" are used. When dilute

solutions of diphtheria toxin are neutralized *in vivo*, relatively more antitoxin of low "avidity" is necessary for complete neutralization than would be predicted from *in vitro* tests carried out using more concentrated solutions. In our opinion the dissociation of the toxin-antitoxin complex upon dilution provides a reasonable explanation for the large discrepancy between the *in vitro* and *in vivo* neutralization of scarlet fever toxin by antitoxin noted by Rane and Wyman (10). Rane and Wyman found 60,000 s r d neutralized per unit of antitoxin in the flocculation test rather than the expected *in vivo* neutralization of only 50 s r d. In the latter case toxin and antitoxin are present in fairly high dilution. If the dissociation constants are large, it follows from the

TABLE IV

Skin Tests on Supernates from Floccules at the Flocculation Point

I Toxin and antitoxin concentration at flocculation point	II Skin test doses per cc of mixture	III Skin test doses* found in supernate per cc	IV Total toxin antitoxin complex found in super- nate at flocculation point
<i>units per cc</i>			<i>per cent</i>
10	300,000	30,000	10.0
40	1,200,000	30,000	3.0
60	1,800,000	20,000	1.0
120	3,600,000	40,000	1.0
180	5,400,000	40,000	0.7
475	13,000,000	80,000	0.6

All mixtures were set at 38°C for 3 hrs, then at 6°C for 18 hrs

* These values were determined in the skin of rabbits. They represent averages of tests on three rabbits.

A curious observation was made during these tests. Although the reactions after 24 hours were similar, after 4 days those from the supernates with 180 units or less per cc had faded, but those from the 475 unit supernate persisted with increased intensity. There was no increase in size.

mass law that an excess of antitoxin must be used if neutralization is to be complete. In the flocculation reaction last traces of toxin need not be neutralized. We have found 30,000 s r d neutralized by one unit of antitoxin in the flocculation test but do not regard this difference from 60,000 as particularly significant in view of the difficulties of skin testing in rabbits.

As we have already pointed out in the introduction, before the flocculation reaction can be used for predicting the potency of pure scarlet fever toxin and antitoxin, it is necessary to prove that the reaction is a specific one between the toxin itself and antitoxin. From an examination of Table II it will be noted that within the region of maximal precipitation (*i.e.* 500 to 740 Lf toxin with 600 units of antitoxin) less than 1 per cent of the toxin was found in the supernatant by intracutaneous rabbit test. Outside the equivalence zone, no

apparent neutralization occurs when tested by the intracutaneous method, presumably because of dissociation. That the precipitation of the toxin is not due to non specific absorption by the precipitate is indicated by the fact that even twice as much specific egg albumin anti egg albumin horse antibody precipitate formed in the presence of scarlet fever toxin, failed to carry down the toxin (Table II). We feel that these demonstrations of the specific precipitation of scarlet fever toxin by antitoxin in the equivalence zone are conclusive evidence that the reaction we are dealing with is in fact specific.

Evans and Gottschall (11) and Bunney and Koerber (12) have recently reported failure to obtain adequate correlation between the flocculation titer and the *in vivo* potency of scarlet fever toxins. It seems worth while to discuss possible reasons for the discrepancy in view of the conclusion drawn above. The fact that the present reaction is specific is not proof of its usefulness as a general method of assay, and does not mean that other flocculating antibodies do not occur in antitoxins made by immunizing with scarlet fever toxins produced on infusion and peptone media. Rane and Wyman (10) note that occasional serums showed double zones with certain toxins, of which only one zone appeared to be specific. Bunney and Koerber (12) found that concentrates of infusion broth or peptone gave flocculation reactions with certain scarlet fever antitoxins. We have confirmed the observation of Bunney and Koerber that flocculation may occur with peptone and antitoxic horse serum. However, after removal of the non specific antibody by flocculation with concentrated peptone solution, the antitoxin still flocculated to titer with scarlet fever toxin. Bunney and Koerber also reported that they were unable to flocculate toxin produced on a casein hydrolysate medium. Using a particularly rapidly flocculating antitoxin we were able to flocculate some of this casein hydrolysate toxin kindly sent us by Dr. Bunney and Dr. Koerber. The toxin which they sent us contained 200,000 s.t.d. per cc. and flocculated slowly at 7 Lf per cc.

While the non specific reactions discussed above may account for some of the failures to obtain satisfactory *in vivo in vitro* correlation it seems unlikely that this can be the whole explanation of Evans and Gottschall's results since they tested their toxins over a wide range and report only one zone of flocculation. There is another possible explanation for the observed discrepancies in titrating scarlet fever toxins. Hooker and Follensby (17) have demonstrated that certain scarlet fever strains of streptococcus, including the NY5 strain, may under certain conditions produce two erythrogenic toxins, A and B. The B toxin is more labile than and immunologically distinct from the ordinary Dick toxin. The presence of two immunologically dissimilar skin toxins would, of course, interfere with their assay by the flocculation test. In our own experience, we have encountered no difficulty in following the purification of toxin from both NY5 and No. 594 strains by means of the flocculation reaction.

Presumably, under our conditions for growing the organisms, no appreciable amount of the Toxin B of Hooker and Follensby was produced

The studies of Rane and Wyman (13) and Plummer and Fraser (14) have suggested that the toxin produced by strain No 594 is identical in its immunological behavior to NY5 toxin. The former has not been accepted as yet by the Scarlet Fever Committee. Table II and Fig 1 show that the No 594 and NY5 toxins are quantitatively identical in the flocculation reaction within the limits of experimental error. Because the No 594 strain produces several times as much toxin as the NY5 strain under similar conditions, the former strain should prove useful for toxin production.

Since skin tests on the supernatants from the equivalence zone show that at least 99 per cent of the toxin-antitoxin complex is precipitated from 600 units of antitoxin by 500 to 740 Lf of toxin, it is possible to calculate the nitrogen per Lf unit for pure scarlet fever toxin from the slope of the quantitative flocculation curve within this region. From three titrations on two different antitoxins, careful nitrogen determinations indicated that pure scarlet fever toxin contains 0.00023 mg nitrogen per Lf unit, a value which we believe to be accurate to within at least 20 per cent. This nitrogen per Lf unit does not have the same significance as in the case of diphtheria toxin because there is no standard flocculating antitoxin available for comparison. It may also be calculated from the data in Tables II and III that pure scarlet fever toxin contains very close to 1.3×10^8 skin test doses per mg of nitrogen. Similar calculations indicate that pure scarlet fever antitoxin contains 0.00093 mg nitrogen per unit. Since the concentrated and partially purified No 594 toxin contained 0.00054 mg nitrogen per unit and 5.7×10^7 s t d per mg nitrogen, we estimate that the preparation contains about 43 per cent scarlet fever toxin assuming that the toxin has the same nitrogen content as protein. Similarly, the NY5 preparation contains about 66 per cent scarlet fever toxin.

From Table III it will be noted that with two exceptions the maximum antitoxin nitrogen precipitated by toxin from 600 units of antitoxin varied between 0.552 and 0.612 mg. The two exceptions are antitoxins No 47E and No 24739. Antitoxin No 47E (National Drug Company) had been treated with pepsin according to the procedure of Pope (19) and from its low precipitable nitrogen it may be assumed that a splitting of the molecule has occurred analogous to pepsin-treated diphtheria antitoxin (9, 20). Antitoxin No 24739 (Squibb Laboratories) was a concentrated pseudoglobulin preparation which had not been subjected to the action of pepsin. We are unable to explain its low specifically precipitable nitrogen content at this time.

According to the above calculations, most of the preparations of scarlet fever toxin which have been reported in the literature were of a very low order of purity. We feel that detailed chemical studies on such preparations should be viewed with some skepticism. Thus the recent work of Barron, Dick, and

Lyman (7) should be interpreted with caution, since according to our calculations, their preparation contained only about 0.2 per cent actual toxin. On the other hand, it seems likely that the preparations of Stock (6) which contained 10^8 s.t.d. per mg. of nitrogen, were of a very high degree of purity. In agreement with Stock (6) and Barron, Dick, and Lyman (7) our results suggest that scarlet fever toxin is a protein. Our preparations, estimated to be about 45 per cent and 66 per cent pure, contained 14 per cent and 12.5 per cent nitrogen respectively and gave strongly positive protein tests in dilute solution. We also feel that the striking qualitative resemblance of the scarlet fever toxin-antitoxin reaction to other protein-antiprotein flocculation reactions, in the horse, namely diphtheria toxin-antitoxin (9), egg albumin-anti-egg albumin (21), and hemocyanin-antihemocyanin (22), constitutes strong supporting evidence for the conclusion that scarlet fever toxin is a protein. In agreement with other workers we have found scarlet fever toxin to be a protein of unusual resistance to action of the proteolytic enzymes pepsin, trypsin, and papain.

The ratio of antitoxin nitrogen to toxin nitrogen at the flocculation point is 4:1:1 for the scarlet fever system (Tables II and III). This is somewhat higher than the corresponding ratio of 3:6:1 found for the diphtheria toxin-antitoxin complex, and in our opinion suggests that the nitrogen content of scarlet fever toxin is lower than that of diphtheria toxin. Both Stock's purest preparations (6) and our own contain only 11 to 14 per cent nitrogen. It therefore appears likely that the composition of the floccules at the flocculation point is almost exactly the same for the two systems. The molecular composition at the flocculation point for the diphtheria toxin-antitoxin and egg albumin-anti-egg albumin (horse) systems corresponds respectively to TA_2 and EaA_2 (21). If now we assume that scarlet fever antitoxin has the same molecular weight as diphtheria antitoxin (180,000) and that the molecular composition of the specific precipitate with toxin is the same for the two systems, then it may be predicted that the molecular weight of scarlet fever toxin will be found to be about the same as that of diphtheria toxin, i.e. about 70,000 (20, 23).

SUMMARY

1. Highly purified scarlet fever toxin has been prepared from culture filtrates of two scarlatinal strains (NY5 and 594B) of hemolytic streptococcus grown on a medium of defined composition.

2. The flocculation reaction of Rane and Wyman has been studied quantitatively and has been shown specific for scarlet fever toxin and antitoxin.

3. Scarlet fever toxin from strains NY5 and 594B are quantitatively identical in their immunological behavior.

4. Pure scarlet fever toxin contains 0.00023 mg. nitrogen per flocculating unit and close to 1.3×10^8 skin test doses per mg. nitrogen as calculated from

the immunological data Both the immunological and the analytical data suggest that scarlet fever toxin is a protein Similar calculations indicate that scarlet fever antitoxin contains 0.00093 mg nitrogen per unit

5 The scarlet fever toxin-antitoxin complex is readily dissociated in dilute solutions In this respect the scarlet fever toxin-antitoxin system contrasts sharply with the diphtheria toxin-antitoxin system

6 The scarlet fever toxin-antitoxin reaction is discussed in relation to other flocculation reactions

Since this paper went to press further evidence for the specificity of the scarlet fever toxin antitoxin flocculation reaction has appeared H Proom (*J Path and Bact*, 1941, 53, 39) has succeeded in recovering scarlet fever toxin in 5 to 10 per cent yield from washed toxin-antitoxin flocules by tryptic digestion

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CAPACITY OF PLEUROPNEUMONIA LIKE MICROORGANISMS TO GROW ON CHORIOALLANTOIC MEMBRANES

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In recent years, the group of microorganisms resembling the etiologic agents of pleuropneumonia of cattle and of agalactia of sheep and goats has attracted considerable interest, particularly because several strains have been recovered from such commonly used laboratory animals as mice, rats, and guinea pigs and from certain non animal sources (Klieneberger (1)) Sabin (2) demonstrated that many laboratory mice carry these microorganisms in or on their conjunctivae and nasal mucosa as apparently harmless parasites. By instilling exudates from rheumatic and other patients into the lungs of normal Swiss mice, Brown and I (3) induced pneumonia in a large proportion of animals, and from these pneumonic lungs recovered pleuropneumonia like microorganisms belonging to Sabin Types A, B, or C. Because of Sabin's discovery of the wide-spread carrier state of mice in respect of these microorganisms, it seemed highly probable that the human exudates did not contain them, and several observers including ourselves, have failed to grow them directly from rheumatic exudates or tissues (4-7).

During our investigations, dropped chorioallantoic membranes of incubated hens' eggs were employed in searching for suspected etiologic agents, and in one instance, pleuropneumonia like microorganisms, recovered from the lungs of intranasally inoculated mice apparently grew in these membranes. This strain was later found by Sabin to belong to his Type C. About the same time, Sullivan and Dienes (8) reported that they were unable to grow a strain of pleuropneumonia like microorganisms on living chorioallantoic membranes, but they could obtain growths when the embryos were chilled to death at 4°C. This strain was identified immunologically by Sabin as his Type A. Almost simultaneously Findlay, MacKenzie, MacCallum, and Klieneberger (9) recorded that a strain of pleuropneumonia like microorganisms, then designated as L₇, grew well on living chorioallantoic membranes.

These discrepancies in the experience of different experimenters suggested that the ability of the pleuropneumonia like group of microorganisms to grow on chorioallantoic membranes be tested, and this communication is the result of such an investigation.

EXPERIMENTAL

Media and Methods

Chorioallantoic Membranes—The chorioallantoic membranes of white Leghorn eggs, incubated 10 to 15 days, usually 11 or 12, were dropped with the usual Good-pasture technique (10), following the drilling of a small hole in the shell immediately over the embryo. The shell membrane was nicked with an iridectomy knife and through this hole the inoculum in 0.1 cc volume was introduced by means of a tuberculin syringe and a fine hypodermic needle. In case the membrane had not dropped, the needle was plunged about 1 cm into the egg. The holes were sealed with a hot sterile vaseline-parawax mixture. Part of the eggs were incubated at 37°C without other manipulation. The rest were chilled to kill the embryos. In the earlier experiments the eggs with dropped membranes were placed in the icebox at 5 to 8°C, for one-half to one hour, but so many embryos thrived in spite of this cooling that subsequently the eggs were chilled by placing them on paper which was resting on CO₂ ice in a heavily insulated refrigerator. When placed directly on CO₂ ice, many of the shells cracked. Because an occasional embryo survived even this refrigeration, the chilling process was finally applied for 1 hour, half before and half after inoculation, with subsequent candling to check the viability of the embryos.

Inoculum—In a few instances, 30 per cent ascitic dextrose broth cultures were employed, but the usual inoculum was an ascitic broth suspension of ascitic agar cultures, it was prepared as follows. From heavy growths on ascitic agar in Petri dishes, several strips embracing a total area of 3 to 5 sq cm were cut out with a sterile platinum spud and transferred to a pointed centrifuge tube, the agar was finely minced with the spud, then mixed thoroughly with 1 to 1.5 cc of 30 per cent ascitic broth. After standing a few minutes, the tube was centrifuged at low speed for 4 minutes. The supernatant fluid was decanted onto the higher side of a slightly sloping Esmarch dish, in this way many small bits of suspended agar were caught on the glass, and the fluid which collected at the bottom of the slope could be easily aspirated through a fine needle into a Luer syringe. Even with these precautions, many minute particles of agar were carried along with the inoculum. The microorganism content of this inoculum was roughly determined by inoculating a 30 per cent ascitic agar plate with a drop of the fluid from the syringe, and determining the amount of growth after 4 days incubation.

Media for Growing Pleuropneumonia-Like Microorganisms—In our hands, 30 per cent ascitic agar and 30 per cent ascitic broth have proven uniformly the most satisfactory media for growing all strains of pleuropneumonia-like microorganisms that have been available. The broth was an infusion of beef heart made in a proportion of 450 gm of lean meat per liter of water, neopeptone 1 per cent and NaCl 0.5 per cent. The final pH was 7.8. To this broth was added 30 per cent of suitable ascitic fluid, or other sera, after it was filtered through a Seitz E K pad under 20 lbs pressure. In some instances, the media was further enriched with 0.5 to 1 per cent dextrose in sterile solution. After incubation to test sterility, the ascitic broth was stored in the refrigerator.

The 2 per cent nutrient agar was prepared from beef heart infusion with 1 per cent neopeptone and 0.5 per cent NaCl and adjusted to a final pH of 7.8. To flasks of this

agar melted and cooled to 45°C was added 30 per cent of Seitz filtered warm ascitic fluid. This mixture was poured into clear glass Petri dishes having *fine filter paper* in the tops to absorb condensed water. When the media was well solidified the Petri dishes were sealed with parafilm in strips about 2 cm wide pressed well on the glass with a photograph squeegee roller. After 24 to 36 hours incubation the plates were examined microscopically and stored on a laboratory table (not in the refrigerator). Before being inoculated the medium was again carefully examined either with a hand lens or low power microscope and those plates showing bacteria or spores of moulds were discarded. It was important to use plates not over 7 to 10 days old because of the tendency of older ones to develop numerous pseudocolonies of spherocysts (11).

These plates were inoculated in a bacteriological hood or in a tissue culture room having filtered air and resealed with fresh parafilm. This seal was an important feature of the technique for when properly made it insured a moist atmosphere in the Petri dish during both the preliminary incubation and storage of the media and following the inoculation.

The material to be tested for content of pleuropneumonia like microorganisms consisted of a pulp of the chorioallantoic membrane or embryo minced finely with a scissors. This was streaked over the surface of the media with the convex surface of a slightly curved spud, which was more satisfactory than the usual platinum loop. In making subcultures a suitable area of the agar was cut out lifted to a fresh plate with a spud and the colony bearing surface of the 'cutout' was placed in contact with the upper surface of the fresh medium then the 'cutout' was carefully rubbed over the surface of the ascitic agar with the spud. The tendency for pleuropneumonia like microorganisms to grow down into the agar makes necessary this mode of transferring described by Klieneberger (12) for it is difficult and often impossible to transfer this type of colony by the usual bacteriological technique. In subculturing these cultures into liquid media a cutout of the agar bearing colonies was transferred to the ascitic broth with the spud. When subcultures were made from fluid to solid media a drop of the ascitic broth culture was placed on the surface of the ascitic agar and the cover of the Petri dish was immediately replaced then the drop of fluid was spread over the surface of the medium by properly tipping the dish with care not to allow the fluid to touch the sides. In all of these maneuvers it was important to reduce the period during which the plates were opened to the shortest possible interval, for air borne bacteria and spores of moulds were frequent sources of contamination, and once contaminated with moulds it was very difficult to purify the cultures of pleuropneumonia like microorganisms without resorting to filtration.

The advantage of the above described technique is that the growth and development of the colonies can be followed with the low power microscope (objective 10X, ocular 10X) without opening the Petri dish and running the great risk of contamination, for the characteristic colonies usually do not become visible before the 3rd day, and sometimes not until the 5th to 7th particularly when grown from tissues. For the most satisfactory microscopic examination, a fairly strong artificial light is desirable, the substage condenser is elevated as high as possible and the beam of light made very fine by almost closing the iris diaphragm. The Petri dish is placed with the top down on the microscope stage and a fine beam of light is focused sharply on the filter paper in the Petri dish. This gives about the proper illumination of the colonies.

which are viewed through both the bottom of the dish and the agar, hence the importance of having these light-transmitting media crystal clear. By slightly tipping the substage mirror from side to side, or by moving the lamp, the beam of light can be made to strike the colonies obliquely and thus their texture and the character of the centers can be better brought out. These colonial appearances are rather characteristic for the different types, as emphasized by Klieneberger (12). Small areas on the medium can be identified by marking the overlying surface of the Petri dish with a glass marking pencil, and isolated individual colonies can be marked by fastening a bit of perforated paper directly over the colony with Scotch tape, if the marker is properly placed, the colony can be repeatedly studied through the perforation, and numbered paper can be used to identify a particular area or colony for transfer.

Obviously for this technique the media must be crystal clear, hence blood agar or Klieneberger's special media (13) for growing pleuropneumonia-like microorganisms is not usable, even though it efficiently nourishes these microorganisms. Neither is clear media slanted in tubes so satisfactory, because of the difficulty in microscopic examination, which is necessary to detect such minute colonies. In our experience, the most important component of this media is proper ascitic fluid in 30 per cent concentration. Not all ascitic fluids are satisfactory, hence it is advisable to test each new fluid for its capacity to sustain all available pleuropneumonia-like microorganisms before adopting it for routine use. With these precautions, we have grown all of the strains of pleuropneumonia-like microorganisms that have come to our hands even though several of them had been grown by Klieneberger for many months on media enriched with horse or beef serum. For many strains, horse-, beef-, or rabbit serum agar are satisfactory enriching substances, but because ascitic fluid in 30 per cent concentration has been found requisite to grow others, it seemed desirable to adopt this as a routine media for recovering pleuropneumonia-like microorganisms from tissues. With it we have easily recovered strains directly from pneumonic mouse lungs, from apparently normal mouse conjunctivae, and from the chorio-allantoic membranes used in the present work. Occasionally, only 3 to 10 colonies could be found on an entire plate, numbers that could easily have been overlooked on any other media. Moreover, it has been possible to obtain subcultures from "cutouts" containing one or two colonies. Finally, the relative number of colonies on a plate inoculated with minced tissue has given a rough index of the number of microorganisms in that tissue compared with others.

Microscopic study of pleuropneumonia-like microorganisms is most easily made in Giemsa stained films and in dark field preparations of fluid cultures such as the 30 per cent ascitic broth, but it sometimes takes much longer to establish cultures of these microorganisms in fluid than on solid media, and then only after many "blind transfers." Moreover, some strains die much more rapidly in fluid cultures than on solid media, and contaminants can be more readily separated when they appear on agar, although it is fairly easy to purify fluid cultures by suitable filtration.

Strains of Pleuropneumonia-Like Microorganisms Tested—Eight different strains belonging to four different immunological types were tested for their ability to grow on chorioallantoic membranes.

L₁ Strains—*L₁* obtained from Miss Klieneberger was originally isolated from a rat's enlarged lymph node. It had been carried through many subcultures, on various media.

L₇ and L_{7b}, obtained from Dr Angevine, were derived from the L₇ strain isolated from arthritic joints of rats by Findlay, MacKenzie, MacCallum, and Klieneberger (9). Later Klieneberger (1) determined that this strain was immunologically similar to L₄. Dr Angevine had repeatedly subcultured the two variants in different media: the *a* variant on ascitic agar and the *b* variant in ascitic broth, and had found different colonial forms in these variants when they were subcultured on ascitic agar.

L₈ Strains—L₈, obtained from Klieneberger, isolated by Findlay, Klieneberger, MacCallum, and MacKenzie (14) from the brain of a mouse with rolling disease.

Sabin Type A—Strain A78 isolated from mice by Sabin (15, 16). Young cultures or filtrates of young cultures, induced rolling disease in mice and cultures inoculated intravenously induced polyarthritis.

Strain CA 95, isolated by the author from pneumonic lung of a mouse which had received intranasal instillations of a rheumatic exudate culture agglutinated in high titre in Type A serum. Klieneberger (1) has shown that Sabin Type A and her L₈ are similar immunologically.

Sabin Type B—Strain B43 obtained from Sabin as a representative strain of this type was isolated originally from Rockefeller Institute stock of albino mice.

Pleuropneumonia contagiosa bovis—Strain P₁ was originally from London type collection, but had been maintained many years on artificial media. Recently kept in desiccated state after freezing.

All of the strains when grown on 30 per cent ascitic agar showed on microscopic examination colony forms of isolated colonies similar to those described and pictured by Klieneberger (12).

RESULTS

The results of testing the four different types, embracing eight different strains or variants, are given in detail in Table I. There were eighteen satisfactory experiments, but in many others in which bacterial contaminants or moulds rendered estimation of the comparative growth of pleuropneumonia like microorganisms on the ascitic agar plates less accurate, the same tendencies were noted.

Under the experimental conditions, three categories of embryonic growth conditions are classifiable: A, those with embryos alive both at the times of inoculation and final examination, AD, those alive when inoculated but dead when examined, and D, those dead when inoculated. Group A included a few where the chilling did not kill the embryo, which then lived and developed like their unchilled mates. In group AD, most of the embryos died shortly after the membranes were inoculated, hence from the standpoint of furnishing living or dead tissue for growing pleuropneumonia like microorganisms, they must be considered almost comparable with group D.

It is unlikely that the pleuropneumonia like microorganisms killed these dead embryos, for if these infectious agents were in themselves lethal, it is improbable that over three fourths of the total inoculated living embryos would have survived. The fact that there is usually some mortality among embryos

TABLE I

Type of pleuropneumonia-like microorganism	Strain	Exp No	Media in which inoculum for embryonic growth was grown	Categories of embryos				D			
				A		AD		Dead when inoculated		Dead when examined	
I ₄	I ₄	14	AA†	—	++	++	±	++	++	++	++
	"	15	AA	—	++	++	++	++	++	++	++
	"	18	AA	++	++	++	++	++	++	++	++
	"	20	AA	++	++	++	++	++	++	++	++
	L _{7a}	31-2	AA	++	++	++	+	++	++	++	++
	L _{7b}	33-4	AA	++	++	++	++	++	++	++	++
L ₈	L ₈	12-13	AB	—	—	—	±	++	++	++	++
	"	19	AA	—	—	—	±	++	++	++	++
	"	21	AA	(±)	—	—	±	++	++	++	++
	A ₇₈	16-17	AA	—	(±)	—	±	++	++	++	++
	CA 95	37	AA	(±)	—	—	±	++	++	++	++
	"	38	AA	—	—	—	±	++	++	++	++
Sabin B	B43	22	AA	±	++	++	++	++	++	++	++
<i>Pleuropneumonia contagiosa bovis</i>	P ₁	23	BB	++	++	++	+	++	++	++	++
	"	24	AA	±	++	++	++	++	++	++	++
	"	25	AA	++	++	++	++	++	++	++	++
	"	26	AB	+	++	++	++	++	++	++	++
	"	27	AA	±	++	++	±	++	++	++	++

† AA = ascitic agar

AB = ascitic broth

BB = beef serum broth

* Growth formed a solid band

(±) to ++++ indicates relative amount of growth on ascitic agar plates inoculated with minced chorioallantoic membranes

— indicates no growth on ascitic agar plates inoculated with minced chorioallantoic membranes

in eggs submitted to the membrane dropping technique probably accounts in large part for the deaths in group AD

In five eggs belonging to group A, the inoculum was injected into the eggs because it was impossible to drop the membranes satisfactorily, as the recovery of pleuropneumonia like microorganisms in all such instances was comparable to that from dropped membranes the results are combined in the table. From blood free allantoic or amniotic fluid of several inoculated eggs, it was possible to grow pleuropneumonia like microorganisms in concentrations almost as heavy as from the corresponding membrane. It therefore seems probable that all tissues bathed by these infected fluids contained these microorganisms.

A casual glance at Table I reveals that there was no general rule concerning the ability of pleuropneumonia like microorganisms to grow on living chorio-allantoic membranes. Three of the four types tested grew well under such vital conditions, but the fourth, Klieneherger's L₄, usually did not survive. Nevertheless, from four out of fourteen living membranes inoculated, respectively, with three different representatives of Type L₄, it was possible to recover a very few colonies on ascitic agar plates. These few colonies would doubtless have escaped detection with any other technique than that which made possible a thorough microscopic examination of the entire agar surface. Whether this slight infection of the membranes would have been detectable by a series of "blind passages" through fluid cultures, it is impossible to state. Certainly it would have been difficult to make such comparative studies with fluid media. One may properly question whether in these exceptions with Type L₄, the survival of the few microorganisms in the membranes might not have been due to their presence in bits of agar carried along in the fluid with which the eggs were inoculated. No data are available to throw light on this question, although with other types good growths occurred on living membranes inoculated with ascitic or beef serum broth cultures free of agar. It is interesting that strains L₄, isolated by Klieneherger, A78, isolated by Sabin, and CA 95, isolated in our laboratory, all behaved similarly in their growth on chorioallantoic membranes, and also much like the Sabin Type A strain, studied by Sullivan and Dienes (8). Their similarity in this respect is confirmatory of the type relationship of these strains, established immunologically by Klieneherger and Sabin, respectively.

From living membranes inoculated with strains belonging to Klieneherger Type L₄, Sabin Type B, and *Pleuropneumonia contagiosa bovum*, good growths were obtained, therefore in sharp contrast with Type L₄, it is obvious that the majority of types tested were capable of growing on such membranes. This capacity, however, varied; it was less marked in the strains belonging to Types L₄ and B than in *Pleuropneumonia contagiosa bovum*, which incidentally induced the most marked macroscopic and microscopic evidence of growth in these viable membranes.

Even though these three types grew well in living membranes, they multiplied in all experiments much better in dead ones. This is best illustrated by reassembling the data in the form shown in Table II, where it is evident that the best membranes for growing all strains were those which were dead when inoculated, *i.e.* category D, next in order were those that died shortly after being inoculated (AD). Indeed, from several of the killed membranes inoculated with strains belonging to strains L₇ (Type L₄) and *Pleuropneumonia contagiosa bovis*, there was obtained on the ascitic agar plates a growth so heavy that it formed a grayish band on the surface of the medium, and the typical colonies were recognizable only on thinly inoculated areas or on subcultures. From these results, it seems evident that if the chorioallantoic

TABLE II

Type of pleuropneumonia like microorganism	Category	Total	Degree of growth on ascitic agar plates						
			-	(±)	±	+	++	+++	++++
L ₄	A	12	2				8	2	
	AD	7							
	D	12							
L ₅	A	14	10	3	1	1			4
	AD	1							
	D	10							
Sabin B	A	5			1	1	2	1	
	D	1							
<i>Pleuropneumonia contagiosa bovis</i>	A	11			1	1	2	3	4
	AD	5							
	D	7							

membrane technique is to be employed in searching for the presence of pleuropneumonia-like microorganisms in tissues or exudates, a better chance of recovering them would be afforded by killing the embryos aseptically before placing the suspected material on the membranes.

Incidentally, the effect of growing variants of pleuropneumonia-like microorganisms on chorioallantoic membranes was studied. This was suggested by some observations of Dr. Angevine (17) from whom strains L_{7a} and L_{7b} were obtained. The L₇ strain had been sent to him by Miss Klieneberger. In culturing the strain on ascitic agar and in ascitic broth respectively and occasionally comparing the morphology of the colonies in subcultures on ascitic agar, Angevine noted that variants developed in the liquid media, which tended to retain their variant morphology when repeatedly subcultured on solid media. When, on the other hand, their pathogenicity was tested in animals, the colo-

nies obtained from the lesions were always of the original type, here designated as *a*, the variants which developed in ascitic broth are designated as *b*. In six subcultures on ascitic agar in our laboratory, the two strains retained their respective colonial appearance. The *a* colonies were round, only moderately granular, the margins were distinct, and the central portions were relatively small and fairly translucent. The *b* colonies, on the other hand, were larger and appeared more granular, the margins were somewhat indistinct, the centers were two to three times the size of the centers of the *a* colonies and were distinctly more granular.

Both types of variants grew well on either living or killed chorioallantoic membranes, but infinitely better growth was obtained from the dead ones (experiments 31-2 33-4). The ascitic agar cultures from all of the membranes, however, presented the morphology of the *a* colonies. Thus the passage of variants through chorioallantoic membranes had the same influence in causing them to revert to the original form as had passage through such mammals as rats and mice. The danger of contamination with other strains of pleuropneumonia like microorganisms in passing through eggs is, of course, much less than in passage through rats and mice, which are now known to be frequent carriers of this class of microorganisms.

Lesions Induced in Chorioallantoic Membranes

No constant lesions were observed in the living membranes inoculated with the several strains of pleuropneumonia like microorganisms. Many of the membranes showed gray central areas of thickening which were probably induced in large part by the technical procedures. Only rarely were there small diffusely scattered white spots which resembled somewhat the lesions described by Burnet (18) as pox. About one fourth of the membranes were thin and transparent, half showed varying degrees of edema, in some very marked, but no relationship could be determined between the amount of edema and the intensity of infection as determined by the content of microorganisms. All of the living membranes infected with strain L₇ were from 1 to 3 mm. thick, and had a gelatinous consistency. Half of the membranes infected with *Pleuropneumonia contagiosa bovum* had a similar thick gelatinous or leathery appearance.

Only a few of the markedly thickened membranes were examined microscopically and no attempt was made to study the histological evolution of lesions. It is doubtful whether the abnormal microscopic picture could all be attributed entirely to the action of the microorganisms for the inoculum often contained numerous minute particles of agar which doubtless induced foreign body reactions in the membranes. The edematous membranes showed some increase in mesodermic cells which were widely separated by clear spaces, the gelatinous membranes often revealed a distinctly increased number of fibro-

blast-like cells, and in those membranes infected with strain L₇ there were numerous scattered accumulations of large round cells with a heavily staining nuclei. Those infected with *Pleuropneumonia contagiosa bovis*, on the other hand, although just as abnormal in the gross, showed much less round cell infiltration in the mesoderm. Because of the great variability in both macro- and microscopic appearance, it is doubtful whether any of the alterations had pathognomonic significance. To determine the chorioallantoic tissue reactions to infection with various strains of pleuropneumonia-like microorganisms, a much more systematic investigation should be pursued with inocula free of particulate foreign bodies.

DISCUSSION

As suggested by the literature, considerable variability was found to exist among different pathogenic strains of pleuropneumonia-like microorganisms in their ability to grow on living chorioallantoic membranes. While the present experiments employed only a part of the known strains of this class of microorganisms, it is evident that no universal rule can be laid down concerning the ability of this class of microorganisms to survive under the peculiar avian embryonic conditions that are favorable for so many bacteria and viruses. From our experience with the three strains of Klieneberger L₅ type (Sabin Type A) examined by us and the one strain tested by Sullivan and Dienes, it appears that immunologically related strains probably have similar capacities for growing on chicken chorioallantoic membranes. On the other hand, within Type 4, strain L₇ induced much more marked lesions in the membranes than did strain L₄, even though both grew with comparatively the same facility in these tissues. No relationship, however, could be detected between a given strain's capacity for inducing lesions in mammals and its ability to multiply in chick chorioallantoic membranes. Indeed, the capacity for these mammalian pathogenic microorganisms to induce marked or characteristic lesions in either the chick embryo or membranes seemed to be minimal. It is doubtful whether any of the strains tested had a lethal effect on these chick embryos, for whether they grew luxuriantly or poorly on the membranes, by far the majority of the embryos survived, and showed no characteristic macroscopic lesion when examined.

It is of interest that passage through chorioallantoic membranes caused a morphological variant L_{7b} to reassume its original colonial morphology in the same manner as passage through its normal host, the rat. This observation suggests that the egg embryo passage technique might be more reliable in studying the ability of strains of pleuropneumonia-like microorganisms to assume their characteristic colonial morphology than would passage through rodents, for it is now well recognized that rodents may harbor many different

types of these microorganisms in an apparently latent form, and this carrier state makes it possible to contaminate a given strain of pleuropneumonia like microorganisms with strains of another variety if it is passed through such animals

The rule that sterile killed chorioallantoic membranes support the growth of pleuropneumonia like microorganisms better than living ones is worthy of note, in this respect they behaved more like bacteria than like viruses, which practically always require living cells for their growth. Possibly sterile dead chorioallantoic membranes would furnish the most suitable medium for pleuropneumonia like microorganisms of fastidious growth demands. Certainly killed membranes should always be used along with living ones, if this technique is employed in searching for pleuropneumonia like microorganisms in tissues suspected of harboring them.

SUMMARY

1 Among eight strains or variants, included in four different immunological types of pleuropneumonia like microorganisms, all grew on chorioallantoic membranes, those belonging to Klieneberger's Type L₄ (Sabin Type A) grew very poorly, and those included in three other types grew with varying degrees of vigor

2 In all instances, strains of pleuropneumonia like microorganisms tested grew better on dead sterile chorioallantoic membranes than on living membranes

3 None of the strains tested was in itself lethal for chick embryos

4 No constant macroscopic lesion developed as a result of inoculating chorioallantoic membranes with pleuropneumonia like microorganisms

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DETERMINATION OF THE VOLUME OF THE EXTRACELLULAR FLUID OF THE BODY WITH RADIOACTIVE SODIUM

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In order to secure precise knowledge of the fluctuations of body water it is necessary not only to obtain the concentration of electrolytes in the serum but also to know the volume of fluid in which they are dissolved and the apportionment of these fluids among the various compartments of the body. While there are now available several fairly accurate methods for measuring the volume of the vascular or plasma fluid, attempts to determine the volume of intracellular fluid have not proved too successful. On the other hand estimation of the extracellular volume by means of certain diffusible, non-metabolized substances such as thiocyanate, sucrose, and sulfate have been more fruitful (1-5). Most of these substances are not present in the body normally but resemble chloride in that they are restricted mainly to the extracellular fluids. The principle of the methods employed is not complicated. Some hours after the intravenous injection of the test substance, samples of blood and urine are obtained and the concentration determined in each. Assuming that the substance is uniformly distributed, the amount remaining in the body divided by the concentration in the serum water gives the volume of fluid in which it is dissolved. It appears that these substances are distributed throughout approximately 20 to 25 per cent of the body weight of man (1, 2, 5).

Laviertes, Bourdillon and Klinghoffer (2) have shown that for practical purposes sucrose and sulfate are not suitable for determination of the extracellular fluid because of their speed of excretion. However, sucrose has an advantage in that it is a non-electrolyte and therefore its concentration in the water of serum and of interstitial fluid is probably the same. On the other hand thiocyanate has numerous advantages. It is rapidly distributed through the body, it is slowly excreted and its concentration can be readily determined with accuracy in a small sample. It has several disadvantages in that it enters the red blood cells, attaining a concentration approximately the same as that of serum, and it enters the cells of certain glandular organs. Laviertes and coworkers (2, 6) and Gilligan and Altschule (7) believe that it is 'bound'

somewhat to some relatively non-diffusible substance in the serum, so that the average concentration in the transudate is 100/110 that in the serum

In view of the fact that none of these substances proved to be ideal for the purpose, a substance such as sodium which is normally present in the body and is mainly limited to the extracellular phase, should afford a convenient measure of the volume of this compartment. This can be accomplished readily by determining the volume of body fluid through which radioactive sodium (Na^{24}) is distributed after its intravenous injection. The purpose of this paper is to describe such a method and to report the results of determinations of the sodium space on normal subjects and to compare these results with those obtained by estimating the volume of fluid available for the distribution of sodium thiocyanate.

Methods and Procedures

Radiosodium was prepared in the cyclotron in the Department of Physics of the University of Rochester. It was formed by the bombardment of sodium chloride with deuterons. The reaction was $\text{Na}^{23} + \text{H}_2 \rightarrow \text{Na}^{24} + \text{H}^1$. The chloride isotope was also produced but it has a very short half life of 37 minutes. Radiosodium decays with the emission of free electrons or β -rays, $\text{Na}^{24} \rightarrow \text{Mg}^{24} + e^-$. The half life of Na^{24} is 14.8 hours. The amount of the radioactive isotope present in a sample was determined by measuring its β -ray activity by means of a modified Geiger-Muller counter. The counting tube described by Bale, Haven, and LeFevre (8) was employed. The radio activity measurements were recorded in terms of scale-of-four counts per minute per milliliter. Each sample was corrected for decay of its β -ray activity to the time of the counting of the original radiosodium standard.

The method is based on dilution of a known quantity of Na^{24} (in counts) by the extracellular water. The volume in which the sodium is diluted is found by dividing the quantity of radiosodium present in the body by the concentration in a sample of serum. The quantity in the body at any given time is the quantity injected minus the urinary loss. Here the assumption is made that radiosodium does not diffuse into the cells, but is exclusively limited to the extracellular fluid. It will be shown later that there is an excess of sodium in the body that cannot be ascribed to the extracellular compartment.

The active material (approximately 300 mg) was dissolved in 13 to 20 ml of distilled water and the solution autoclaved. A measured volume (usually 9.16 ml) of this solution was injected into the antecubital vein from a specially calibrated syringe. The syringe was rinsed with blood several times to insure delivery of all the sodium. From the remaining portion of the radiosodium solution, 1 ml samples were delivered into three separate volumetric flasks and diluted with physiological saline to 2 liters. These are referred to as the radiosodium standards. It was found that three samples were necessary for accurate determination of the number of counts injected.

Simultaneously, the thiocyanate method for the measurements of extracellular fluid was carried out as described by Gregersen and Stewart (4). Immediately following the injection of the radioactive sodium, 16 to 18 ml (0.8 to 0.9 gm) of sterile 5 per cent sodium thiocyanate solution were injected slowly into the ante-

cubital vein of the subject from a calibrated syringe. Thiocyanate was determined as ferric thiocyanate by the use of the spectrophotometer in preference to the colorimeter (14). The readings were made at a wave length of 480 millimicrons.

Blood volume determinations were made in some of the subjects by the use of the blue azo dye (T 1824) as described by Gibson and Evans (9). Optical densities were determined by the spectrophotometer at wave lengths of 620 millimicrons for the dye and 574 millimicrons for correction for hemolysis. In order to detect possible change in the relationship of cells and plasma several hematocrits were taken during each experiment. Blood was also taken for plasma protein determinations.

The experiments fall into two groups: those carried out during the daytime ranging from 3 to 6 hours and those conducted at night for a 12 hour period. The subjects were kept under basal conditions. Control serum and urine samples were obtained from each subject prior to the injection of radioactive sodium and thiocyanate. Blood samples were taken at fairly regular intervals during each experiment to determine the mixing time of Na^{24} and thiocyanate. In one group of patients the concentrations of Na^{24} and thiocyanate in blood were compared with those of various serum effusions. Two blood samples (6 ml of whole blood) were taken in separate syringes through one needle. Such samples were put under oil, allowed to clot, centrifuged and the serum pipetted off in preparation for the determination of β ray activity, dye and thiocyanate concentrations. Urine specimens were obtained following the venipuncture in order to determine the excretion of radiosodium and thiocyanate. Duplicate transudate samples were taken immediately following the blood samples.

From each sample of the standard radiosodium solution serum, urine, and transudate a 2 ml aliquot was pipetted carefully into a Geiger Müller cup. These were counted for at least two 5 minute periods. Further periods were often counted to increase accuracy.¹ The optimum range for the Geiger Müller counter used in most of the experiments was below 100 counts per minute for a 2 ml. sample. When the activity of samples exceeded this value time was allowed for radioactive decay until the optimum counting range was reached. An improved counter with a very much higher optimum range was used in the more recent determinations.² The same urine, serum and transudate samples were used for the thiocyanate determinations. No toxic reactions of any kind were observed with either thiocyanate or radiosodium.

¹ The procedure for counting the β ray activity of all the samples for one experiment is as follows: (1) Distilled water background (2) Potassium acetate standard (3) Radiosodium standard number 1 (radiosodium standard number 2 (radiosodium standard number 3) (4) Control serum sample (5) Duplicate serum samples (taken at varying intervals following the injection of Na^{24}) (6) Duplicate transudate samples (7) Control urine sample (8) Urine samples (taken at varying intervals following the injection of Na^{24}) (9) Radiosodium standard (one selected at random) (10) Potassium acetate standard (11) Distilled water background.

² It was found that neither the specific gravity nor any of the constituents of serum and urine had any effect upon the penetration of β rays. Equal amounts of radiosodium solution were added to equal amounts of serum, distilled water and urine and these solutions exhibited the same β ray activity.

The fluid available for distribution of radiosodium (sodium space) was calculated as follows

$$\text{Sodium space, liters} = \frac{\text{Counts injected} - \text{counts lost in urine}}{\text{Concentration counts per minute per liter of serum}}$$

The interstitial fluid (extravascular extracellular fluids) is equal to the sodium space minus the plasma volume

The fluid available for the distribution of thiocyanate was calculated by the formula

$$\text{Thiocyanate space, liters} = \frac{\text{Mg CNS injected} - \text{mg lost in urine}}{\text{Mg CNS per liter of serum}} \quad (\text{Formula A})$$

Thiocyanate space was also calculated according to the formula of Lavietes, Bourdillon, and Klinghoffer (2),

$$\begin{array}{l} \text{Thiocyanate} \\ \text{space} \\ \text{corrected} \end{array} = \frac{\text{Mg CNS retained} - (\text{concentration in serum} \times \text{blood volume})}{100/110 \times \text{concentration in serum,}} + \frac{\text{serum volume}}{\text{volume}} \quad (\text{Formula B})$$

Gregersen and Stewart (4) found that the available fluid inside the red blood cells is equal to their water content, which is about 70 per cent of the cell volume. Furthermore, when the cell volume is calculated from the plasma volume and the hematocrit, it is somewhat higher than the cell volume determined directly by the carbon monoxide method. This formula then attributes too much thiocyanate to the erythrocytes.

Accuracy of the Method—In order to determine the value of this procedure for use in clinical investigation, it is necessary to establish the magnitude of errors inherent in the method. The technical errors resolve themselves into (a) pipetting, sampling, and counting the β -ray activity of the standard, serum, and urine and (b) the measuring and injection of the radioactive substance into the body. There is ample data on the former to describe the limits of errors with reasonable accuracy. All counts referred to in this paper represent scale of-four counts on the impulse recorder and therefore one-fourth the total number of discharges in the counting tube. It was found that three times the standard error of a single determination of β -ray activity was the same for individual standard solutions and serum samples, amounting to 2.3 counts per minute per milliliter. There was no correlation between the magnitude of the error and the degree of activity of the same over a range of 10 to 100 counts per minute per milliliter, therefore, the error in counting a single sample is properly expressed in counts per minute per milliliter rather than in per cent. The error in the sodium space due to counting errors in standard, serum, and urine samples depends, because of this, on the magnitude of activity of the various samples, with accuracy increasing as the activity increases. From the analysis of the data available, the error in the sodium space due to errors in counting the activity of these samples is ± 2520 ml when the count is equal to 10 per minute per milliliter, ± 644 ml when the count equals 40, and ± 266 ml when the count is 100. The average count in this series of experiments was 40 per minute per milliliter. Therefore, with an average sodium space of 18.3 liters, the error, due to counting, should not exceed ± 0.644 liter or ± 3.5 per cent in 95 per cent of the cases.

This takes into consideration most of the technical errors that can be determined, except for the measuring and the injecting of radiosodium. In order to get an idea of the magnitude of error involved in these procedures, a series of five *in vitro* experiments was done. The procedure was as follows: 14 liters of normal saline were placed in a large container, and 9.16 ml of a radioactive sodium solution were injected. The measurement and injection of the sodium were carried out in the same manner as though it was to be injected into a subject. After thoroughly mixing the contents of the container, samples were taken in order to determine the β ray activity. Then 2 more liters of saline were added to the container and the process repeated. The total counts injected divided by the activity of the sample per minute per cubic centimeter gave the estimated volume in liters. The mean difference between the theoretical and the estimated volumes was -0.013 liter with a standard deviation of ± 0.4146 . Correcting for errors of distribution due to the small size of the sample at a level of significance of $P = 0.05$, the deviation of the sodium space from the 'truth' should not exceed ± 0.896 liter (10). The percentage error with an average sodium space of 16 liters is ± 5.6 . Since the average count in this series of experiments was 75 counts per minute per cubic centimeter, the error to be expected from counting the β ray activity should not be greater than ± 0.268 liter. The difference between the values (± 0.896 and ± 0.268 liter) theoretically represents the error in measuring and injecting the radiosodium.

In order to define the limits of error in the thiocyanate method, the volume of fluid in the above experiments was also estimated by means of sodium thiocyanate. These errors include most of the technical ones associated with this method. The mean difference between the actual and estimated volumes was $+0.052$ liter with a standard deviation of ± 0.193 liter. Again correcting for the errors of distribution due to the small size of the sample, the deviation of the thiocyanate space from 'truth' should not exceed ± 0.425 liter in 95 per cent of the cases. This represents a percentage change of ± 2.7 with an average volume of 16 liters.

Material—Twenty-two measurements of the extracellular fluid were made on thirteen normal subjects whose ages varied between 20 and 56 years, body weight between 58 and 106 kg, and body surface area between 1.6 and 2.3 square meters. All the subjects were in good health.

In order to determine the rate of diffusion of radiosodium and thiocyanate from the blood stream into the interstitial fluid, serous effusions into the pleural cavity caused by congestive heart disease, Hodgkin's disease, and pulmonary tuberculosis were obtained ten times from 5 patients; ascitic fluid from a patient suffering with congestive heart failure; synovial fluid from a patient with rheumatoid arthritis; and spinal fluid from four subjects whose fluid showed no abnormalities. Saliva was collected from three normal subjects and gastric juice from two.

RESULTS

Rate of Diffusion of Radiosodium and Thiocyanate—After the intravenous injection of the radioactive isotope of sodium, the β ray activity of the serum gradually fell with time until equilibrium was reached. In Fig. 1 are plotted the volumes of extracellular fluid calculated from the concentration of counts

in the serum against time. These volumes were corrected for loss of radio-sodium in the urine. In general from 1 to 3 hours after the intravenous injection, the volume increased rather rapidly (Fig 1 A), while from 3 to 9 hours

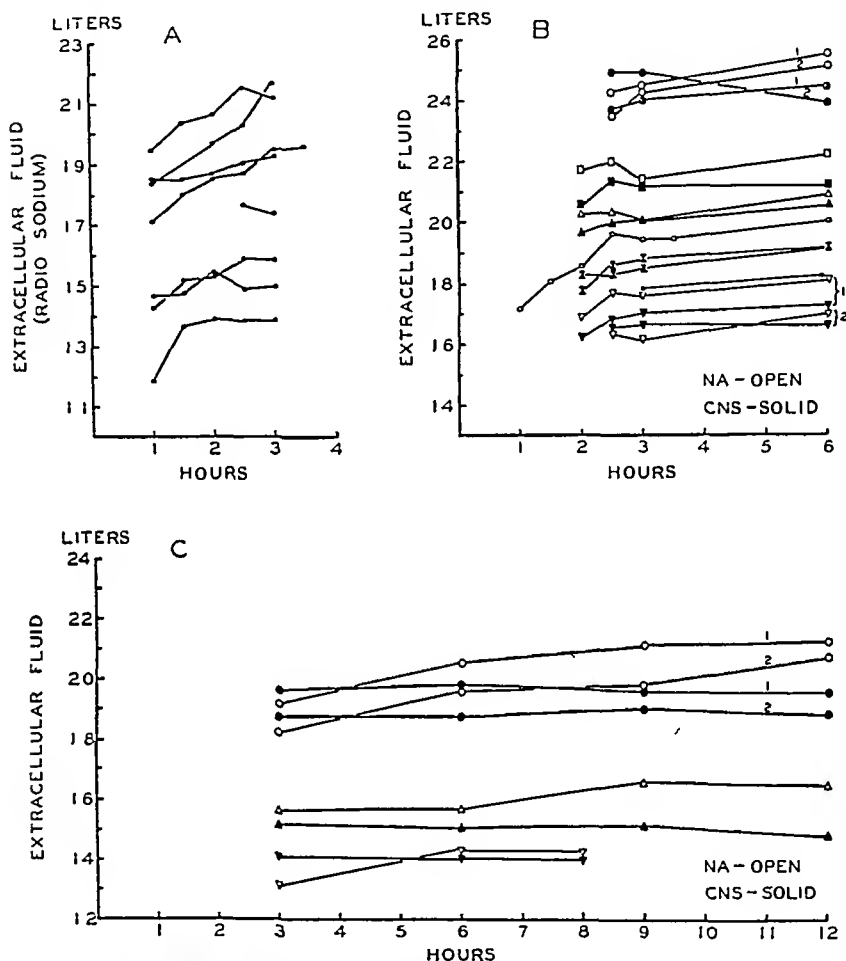


FIG 1 The rate of diffusion of radiosodium and sodium thiocyanate in the body of normal subjects after intravenous injection

after the administration the rate of increase was much slower, indicating that the marked sodium was diffusing more slowly into certain parts of the body (Fig 1 B and C). Measurements of the extracellular fluid on eight normal subjects showed that the average value for the volume 3 hours after the injection was 18.8 liters as compared with 19.6 liters at 6 hours or an average increase of 800 cc. A similar increase was noted from 6 to 9 hours after the

injection, while from 9 to 12 hours the values approximated each other more closely (Fig 1 C). Serial determinations of the sodium space on two normal subjects at 3, 6, 9, and 12 hours gave average values of 17.4, 18.1, 18.9, and 18.9 respectively. The rapidity with which the β ray activity of the serum fell during the first 3 hours after the injection suggests that the tagged sodium diffused rapidly into certain portions of the body (24.8 per cent of the body weight) while the slower rate of decline during the next 6 hours indicates that there are certain portions of the body into which the sodium penetrates less easily.

In some instances the diffusion rate of thiocyanate was studied simultaneously with that of Na^{24} . The rate of diffusion for thiocyanate was more rapid than that for sodium. Equilibrium was reached 3 hours after the administration (Fig 1 B and C). In eight normal individuals the average value for the volume of fluid available for the distribution of thiocyanate was 18.8 liters 3 hours after the injection and 18.9 liters at 6 hours. When samples of serum were obtained every 3 hours for a 12 hour period, the average values for the thiocyanate space (not corrected) in two subjects were 17.4 liters at 3 hours, 17.3 liters at 6 hours, 17.3 liters at 9 hours, and 17.0 liters at 12 hours.

The rate of attainment of diffusion equilibrium for Na^{24} and thiocyanate between the blood stream and serous effusions was also studied. The results are given in Table I. All the subjects had rather large accumulations of fluid. In the case of pleural fluid, diffusion equilibrium for both Na^{24} and thiocyanate appeared to be reached in most cases between 9 and 12 hours after the injection of the substances. This was also true in the case of ascitic fluid (7). 10 liters of ascitic fluid were withdrawn from patient V C. The observations with respect to thiocyanate are not in accord with the results of previous workers (2, 6, 7), who found the concentration of thiocyanate in transudates was 100/110 of the concentration in serum. This discrepancy in results is due in all probability to the fact that the effusions in the cases herein reported are not strictly speaking normal interstitial fluid because of their high protein content.

In contrast to other ultrafiltrates of serum, thiocyanate does not enter the spinal fluid to any great extent. This confirms the observation of Wallace and Brodie (11) that thiocyanate does not enter the central nervous system, unless the concentration in the blood stream is much higher than that used in these experiments. It required about 12 hours for Na^{24} to attain diffusion equilibrium between spinal fluid and serum (Table I). If, as has been suggested (12), the extracellular fluid of the central nervous system has the same relationship to spinal fluid as that of other tissues has to serum, then radio-sodium diffuses slowly into nervous tissue and even 12 hours after injection it may not yet be in ionic equilibrium with spinal fluid. In the dog the radio-

sodium has not penetrated the central nervous system to its maximum concentration (Table V), for the apparent extracellular volume calculated from Na^{24} was smaller (29.3) than that calculated from the chloride (44.6). According to Manery and Hastings (13) the ratio $\text{Na} : \text{Cl}$ is slightly higher for spinal cord than for ultrafiltrate of serum.

TABLE I

Concentration of Radiosodium and Thiocyanate in Serous Effusions Expressed as Percentage of the Serum Concentration

Subject	Serous effusion	Time after injection	Protein		Sodium concentration	Thiocyanate concentration	Condition
			Serum	Effusion			
		hrs	gm per cent	gm per cent	per cent	per cent	
M M	Pleural fluid	3	4.7	2.7	61	101	Hodgkin's disease
	"	6			87	130	
E H	"	6	6.4	4.8	90	87	Congestive heart failure
	"	9			101	99	
	"	12			95	101	
G J	"	6.5	6.4	2.5	103	94	Congestive heart failure
	"	9			99	103	
A W	"	3	6.3	3.0	87	—	Congestive heart failure
	"	6			94	—	
	"	24			97	—	
N H	"	9	8.8	6.3	78	78	Pulmonary tuberculosis
A J	Synovial fluid	12	7.0	5.4	101	95	Rheumatoid arthritis
V C	Ascitic fluid	12	6.3	3.3	95	100	Congestive heart failure
	"	13			97	99	
B L	Spinal fluid	3			22	—	Normal
J O	"	6			23	9	"
M E	"	9			68	—	"
F B	"	12			101	0	"
P M	Saliva	3			7	870	Normal
L Y	"	3			7	1150	
F B	"	3			13	1280	
N H	Gastric juice	3			39	111	
P Q	"	6			28	770	

It has been shown that thiocyanate enters the cells of the glands of the digestive tract, and that it is secreted into the alimentary tract (1). The results of this study showed that the concentration of thiocyanate in saliva and gastric juice was very much higher than that in the serum. On the other hand, the concentration of radiosodium in such fluids was in the expected range (Table I).

Volume of Extracellular Fluid in Normal Subjects—The results of measurements made on fourteen subjects are given in Table II. The average value for the sodium space calculated from the counts in the serum sample taken 3 hours after the injection of labeled sodium was 18.8 liters or 24.8 per cent of

the body weight and 9.7 liters per square meter of body surface area. The extreme values for the volume were 14.2 and 24.4 liters. At 6 hours the average

TABLE II

The Fluid Available for the Distribution of Radiosodium and Thiocyanate in Normal Subjects

Experiment No	Normal subject	Date	Weight	Time after injection	Plasma volume	Extracellular fluid sodium space volume	Extracellular fluid thiocyanate space	
							A	B
		1939	kg	h s	liters	liters	liters	liters
1	H H	Apr	69.6	3	3.16	19.0		
2	J Z	Sept.	67.1	3	2.77†	13.9		
3	J De	Aug	80.6	3	2.82	19.4		
				6		20.0		
4	J D	1940 Mar	64.6	3	3.07	16.7		
				6		17.1		
5	J T‡	June	51.0	12	2.41†	17.5	17.5	17.0
6	J P	Jan	69.8	12	2.58	18.4	17.8	16.8
7	L Y	Mar	84.5	3	3.31	20.1	20.2	18.9
				6		20.9	20.6	19.4
8	F B		102.5	3	2.84	21.3	21.2	20.0
				6		22.2	21.0	19.8
9	V D	Apr	61.3	3	2.99	17.6	17.0	15.8
				6		18.2	17.1	16.0
10	J A	Mar	67.3	3	2.73	17.6	17.8	16.8
				6		18.3	18.2	17.2
11	G M	Apr	106.3	3	4.33	24.4	24.0	22.5
				6		25.5	24.4	22.9
12	S G		56.8	3	2.43†	14.2	15.2	14.3
				6		15.3	15.1	14.2
				8		15.2	15.1	14.1
13	N K		77.6	3	3.86	19.2	19.6	18.1
				6		20.5	19.7	18.2
				9		21.1	19.6	18.1
				12		21.2	19.5	18.0
14	J C	Mar	58.6	3	2.35	15.6	15.2	14.2
				6		15.6	15.0	14.1
				9		16.6	15.1	14.2
				12		16.5	14.7	13.8
Mean‡ standard deviation				3	3.10	18.8	18.8	17.6
					±6.24	±3.04	±2.83	±2.53
Mean‡ standard deviation				6		19.6	18.9	17.7
						±3.22	±2.93	±2.96

* See Methods

† Plasma volume predicted on the basis of surface area from the data of Gibson and Evans

‡ Recent weight loss.

§ Includes Experiments 7 to 14

value for the sodium space was 19.6 liters or 25.9 per cent of the body weight. Subject V D, a tall, slender individual, had the highest extracellular volume relative to his body weight, 29.7 per cent of the body weight, while the individual F B, with an excessive amount of fat, had the smallest volume relative to his body weight, 21.7 per cent. These values calculated from the sample of

serum withdrawn 6 hours after administration of the substance are somewhat too low because diffusion of the marked sodium was not complete at this time 12 hours should be allowed for establishment of complete diffusion equilibrium between serum and tissues

The average value for the interstitial fluid (extravascular extracellular) 6 hours after the injection of radiosodium was 16.5 liters or 21.8 per cent of the body weight 84 per cent of the sodium space was represented by the interstitial fluid and 16 per cent by the vascular compartment

In three normal subjects, N K, J C, and J P, the average value for the sodium space determined from the 12 hour sample was 18.7 liters representing 27.4 per cent of the body weight and 10.5 liters per square meter of surface area

Simultaneous determinations of the fluid available for the distribution of thiocyanate were made on eight of these subjects When the thiocyanate space was calculated simply as the amount of thiocyanate retained in the body divided by the concentration of substance in the serum (Formula A, Table II), the average value was 18.8 liters or 24.9 per cent of the body weight at 3 hours and 18.9 liters or 25 per cent of the body weight at 6 hours The volume of the extracellular fluid calculated according to the formula of Laviertes *et al* (Formula B, Table II) was somewhat lower than that found for the sodium space The average value of eight normal individuals for the thiocyanate space was 17.6 liters and 17.7 liters calculated from the 3 and 6 hour sample of serum respectively If in Formula B we substitute 70 per cent of the total cell volume (its water content) as the amount of fluid available for thiocyanate rather than 100 per cent of the cell volume, then the average values for the thiocyanate space for these eight normal subjects are about the same as those calculated according to Formula A, *i.e.*, 18.7 liters at 3 hours and 18.8 liters at 6 hours In view of this we agree with Gregerson and Stewart (4) and Stewart and Rourke (14) that the best way to calculate the thiocyanate space is according to Formula A These figures for the thiocyanate space are very similar to those reported previously (1, 2)

Repeated Volumes—Repeated determinations of the extracellular fluid by means of both radiosodium and sodium thiocyanate were made on normal subjects at varying intervals of time and under similar conditions In many of these subjects, at the time of the second measurement, a small amount of thiocyanate and radioactive sodium was still present in the body from the first injection In such cases the change in concentration in the serum was used in calculating the volume The results are given in Table III The average difference between the duplicates was -0.43 liter with a standard deviation of ± 0.40 liter for the sodium space Taking into consideration in the calculation the small size of the sample, differences between duplicate determinations of the sodium space should not exceed ± 0.98 liter or ± 4.9 per cent with an average volume of 19.9 liters This error in the sodium space,

cent The mean difference was not significantly different from zero There was no significant change in the blood volume, the mean difference being $+6$ ml The changes in the sodium space were no more consistent than those found for the volume of the blood The volume during the warm season as compared with the cold increased in three subjects and decreased in two The mean difference was -0.50 liter, a value not significantly different from zero

These results are not in agreement with the marked changes in the volume of the blood found by Barcroft *et al* (15) and Bazett and coworkers (16), during seasonal variation The former investigators using the CO method for determining the volume of the blood found an increase of about 35 per cent in this volume as they sailed through the tropics and Bazett *et al* observed equally large increases in the blood volume during the summer beat There was an increase in both the plasma and the cell volume in the summer, so that the hematocrit readings did not change More recently Forbes, Dill, and Hall (17) found much less marked changes in the blood volume with changes in climate A group of laboratory workers on moving to a hot damp climate for the summer showed an increase in the volume of the blood and plasma of only 4.5 and 4.2 per cent, with a range from -6 per cent to $+12$ per cent

We have employed essentially the same methods for the blood volume as Forbes and his group The results in the two groups are very similar The discrepancy between the results of Barcroft and Bazett and Forbes and ours cannot be explained at the present time

DISCUSSION

The results of this investigation indicate that the fluid available for the distribution of radiosodium (sodium space) represents approximately 25 per cent of the body weight The sodium space will be equal to the volume of extracellular fluid providing that either sodium is exclusively limited to the extracellular phase, or if not, it does not exchange with intracellular sodium and further, providing that the radioactive isotope is equally distributed throughout the sodium phase There is good evidence to suggest that neither sodium nor chloride is exclusively limited to the extracellular compartment Manery and Hastings (13) have shown that the Na:Cl ratio varies in different organs The ratio for most tissues is approximately the same as that of an ultrafiltrate of serum, but in certain tissues (stomach, tendon, and testes) there is excess chloride while in certain other tissues (spinal cord, cartilage, and bone) there is extra sodium which suggests the existence of both intracellular sodium and chloride The results of Harrison, Darrow, and Yan net (18) on the analysis of whole bodies of animals support these findings with respect to sodium They found an excess of sodium amounting to ap

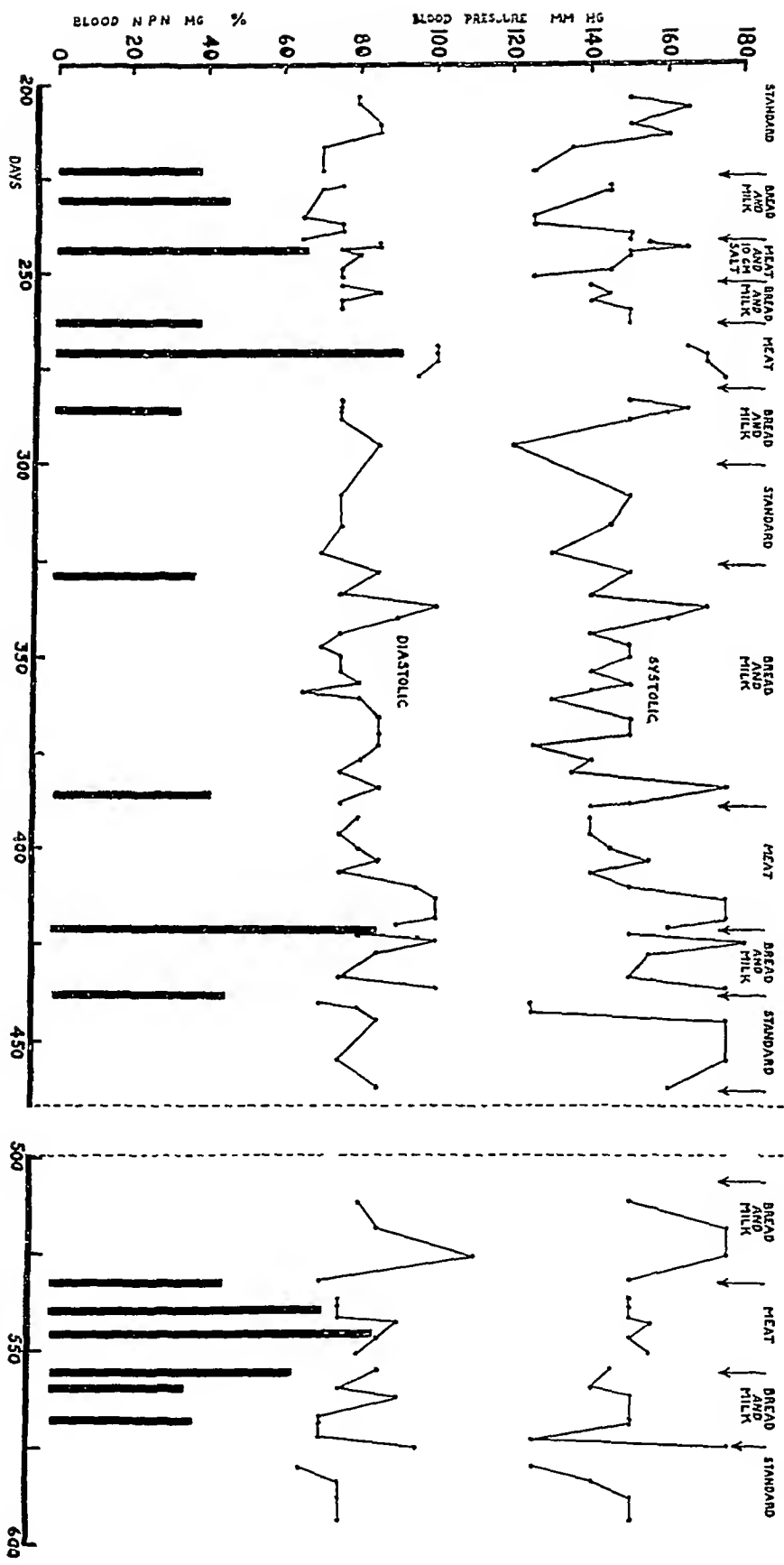


FIG 2

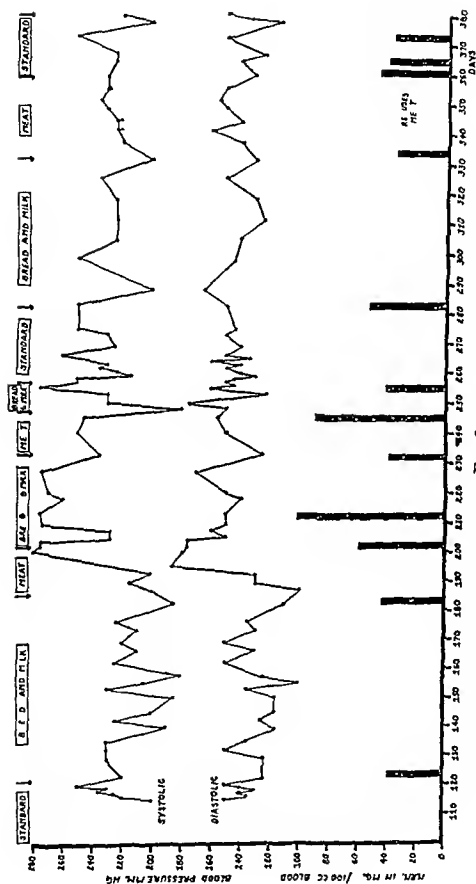


FIG 3

(d) Three dogs (Y-9, X-29, X-53) with mild hypertension and relative renal excretory insufficiency due to partial occlusion of the renal arteries were subjected to the dietary sequence. The non-protein nitrogenous substances of the blood increased during the high protein phase of the diet and returned to the basal level again when the dogs were placed on the standard diet. One of these dogs (X-29) was also subjected to a diet of meat with added sodium chloride, this procedure had no effect on the blood pressure.

(e) Four dogs with severe hypertension and relative renal excretory insufficiency (U-39, X-55, X-64, X-92) were subjected to the succession of high and low protein diets with no significant changes in blood pressure except in one case (X-92). In this animal the first high protein diet was accompanied by an acute rise in blood pressure and non-protein nitrogen as well as symptoms and signs usually associated with malignant hypertension such as anorexia, diarrhea, vomiting, loss of weight, muscular twitching, and corneal infections. In order to ascertain whether these hypertensive and uremic manifestations were causally related to the high protein diet or whether they were coincidental, this animal was twice subjected to a repetition of the diets. In the repeated experiments on this dog the blood pressure began to rise acutely when fed on a high protein diet. When the associated uremic symptoms discussed above manifested themselves along with a concomitant rise in non-protein nitrogen the blood pressure began to fall toward the previous hypertensive level (Fig. 3). The 24 hour urinary concentration test gave a specific gravity of 1.037 and 3 plus albumen was present.

DISCUSSION

In order to judge the significance of our experimental results it is necessary to analyze the interrelationships which may exist between relative renal excretory insufficiency and renal ischemia.

In the normal animal under homeostatic conditions the amount of the excretory work performed by the kidneys is determined by the excretory load placed upon them. The load placed upon each nephron is a function of the concentration and type of metabolic products in the blood stream. The amount of excretory work the kidney can accomplish is limited by its oxygen supply since otherwise a progressively increasing oxygen debt would be incurred and this would of course have an upper limit.

When the blood flow to the kidney is limited, as in nephrosclerosis or partial occlusion of the main renal arteries, an increased load on the kidney may result in relative renal excretory insufficiency or an increase in renal excretory activity sufficient to compensate for the added burden.

The steps involved under these two circumstances can be briefly summarized. With the presence of a decreased rate of blood flow through the kidneys, the blood non-protein nitrogen content as well as other renal excretory products

may rise and thus cause an increased load on the kidney. If the work done by the kidney increases to meet this load but the blood flow cannot increase sufficiently to supply the kidney with an adequate amount of oxygen, pressor products of renal intermediate metabolism can be released into the blood stream in sufficient concentration to cause a generalized increase in the systemic peripheral resistance. An elevated blood pressure will follow. This in turn will cause an increase in the pressure head driving blood through the kidney, thereby leading to an augmentation of the rate of blood flow and of the filtration pressure in the glomeruli. This process would tend to reduce the level of the non protein nitrogenous products and other renal excretory substances in the blood to a minimum. In this way a mechanism may be postulated whereby renal excretory insufficiency may set up the conditions for renal ischemia.

Should the increased load fail to rouse the nephrons to sufficient effort, little change in the metabolic processes of the kidney would occur. The blood flow might remain adequate for the effort. Hence no increase of renal intermediate metabolites would occur and renal hypertension would not ensue. Despite the unchanged blood pressure the rising concentration of the non protein nitrogenous products of the blood would cause by simple physical means an increased elimination of these products through all the excretory organs of the body. In such a manner, a purely physical equilibrium may be achieved and allow the existence of an individual for a period of time in an advanced state of azotemia without hypertension.

Compensation for an inadequate renal function may in time also be aided by the process of renal cellular hypertrophy and hyperplasia. The increase in non protein nitrogenous products in the blood has been reported (10) to cause an increase in the volume of the renal parenchyma by a proliferation of the cellular elements without any change in the number of nephrons.

Obviously, the various processes described above may occur in different degrees and combinations.

The experimental results cited in this report corroborate previous work in this laboratory which emphasizes that renal excretory insufficiency and renal ischemia are separate and distinct processes (14). In addition we suggest a mechanism by which these often concomitant processes may be interrelated.

In only two of our ten dogs (W 77 and X 92) that showed signs of renal excretory insufficiency did the arterial pressure rise during the high protein diet. In the other eight animals compensation for the increased inadequacy of renal function took place either by physical means or by hypertrophy and hyperplasia.

Since both the systolic and diastolic blood pressure of normotensive and hypertensive dogs may show spontaneous fluctuations even under the controlled conditions which were maintained in these experiments, it is necessary

that minor changes in blood pressure be disregarded. It is possible that the amount of change in blood pressure which various workers consider significant may be responsible for some of the discrepancies in the literature (12, 13).

Our results suggest that the elimination for therapeutic purposes of large amounts of protein from the diet of patients with hypertension and little or no renal damage is not generally indicated. Even in patients with hypertension and moderate or greater renal damage, elimination of protein from the diet is probably of little value in the treatment of the elevated blood pressure, although it may be of value in the treatment of the concomitant azotemia.

SUMMARY

The effect of high and low protein diets was studied on fourteen dogs in twenty-four different experiments. In only two of these animals, both with moderate renal excretory failure, was a reversible rise in blood pressure elicited by a high protein diet.

The possible mechanisms involved in meeting an increased excretory load are discussed.

We are indebted to Mr. R. Asher and Miss L. Friedberg for their assistance in these experiments.

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OCCURRENCE OF POLIOMYELITIS VIRUS IN AUTOPSIES, PATIENTS, AND CONTACTS*

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Attempts have been made in the studies on poliomyelitis conducted in this laboratory in recent years, to recover virus from autopsies, from patients, and from contacts. In the earlier work efforts were concentrated on isolation of virus from cord and medulla of human autopsies and cerebrospinal fluid and nasal washings of patients. During the last 2 years other tissues, colon contents, and urine of autopsies and stools of patients and contacts also have been studied. The present report summarizes this work from the time of its beginning in July, 1934, to the close of last season, December, 1940.

Materials and Methods

Tissue—The tissues listed in Table I were collected from autopsies under aseptic conditions and preserved in sterile 50 per cent glycerol with buffered saline at 4°C. They were prepared for injection by grinding in a sterile mortar then adding buffered saline to make a 10 per cent suspension. The suspension was centrifuged and 1 cc. of the supernatant fluid was injected intracerebrally and 3 cc. intraperitoneally usually into *Macacus mulatta*. In a few instances *M. irus* was used and appeared to be just as susceptible as *M. mulatta*.

Cerebrospinal Fluid—Several methods were employed in inoculating spinal fluids. Individual fluids were injected into monkeys either by the intracerebral or intrathecal route, while a simultaneous intraperitoneal inoculation was made with fluids that were in excess. In certain instances the fluids were pooled before injection. Swiss mice also were injected with the same fluids in an attempt to recover other neurotropic viruses.

Nasal Washings—Nasal washings were treated in the following several ways before injection:

1. Treated with 0.5 per cent phenol to destroy the bacteria following either centrifugation or vacuum distillation.
2. Filtered through Berkefeld V filters.
3. Placed in 50 per cent glycerol with saline and kept in the ice box at 4°C for 3 days.

* Aided by a grant from the National Foundation for Infantile Paralysis Inc.

Intracerebral and intraperitoneal injections and intranasal instillations were performed

Stools—Stools were treated for injection both by the etherization method of Trask, Vignec, and Paul (1) and by the intranasal instillation method of Howe and Bodian (2). A high mortality rate followed intracerebral injections and these were discontinued early in the work. About half of the monkeys received both intranasal

TABLE I

Material inoculated	First passage			Second passage		
	Number of individuals	Number positive	Positive	Number tested	Number positive	Positive
			per cent			per cent*
Autopsy						
Spinal cord medulla	72	36	50	33	24	33
Cerebral cortex	21	0	0	0	—	—
Olfactory bulb	11	1	10	1	0	0
Hypophysis	5	0	0	0	—	—
Lymph node (mesenteric)	14	0	0	0	—	—
Thymus	1	0	0	0	—	—
Peyer's patch	1	0	0	0	—	—
Liver-spleen	3	0	0	0	—	—
Bile	6	0	0	0	—	—
Colon contents	19	5	26	3	2	11
Urine	9	0	0	0	—	—
Tonsil adenoid	6	3	50	2	2	33
Living patient						
Cerebrospinal fluid	134	0†	0	0	—	—
Nasal washings	139	0§	0	0	—	—
Stool	53	11	20	7	7	13
Urine	9	0	0	0	—	—
Contact						
Stool	19	1	5	1	1	5

* Per cent positive in second passage is based on the original number of attempted isolations

† Questionable results in 1 case

§ Questionable results in 3 cases

and intraperitoneal injections of the prepared stool material while the other half received intranasal instillations only. The intraperitoneal injections along with the intranasal did not increase the number of isolations accomplished. In fact, in the 1940 series 2 stools out of 16 that were injected by both methods were positive, while 6 out of 21 receiving only the intranasal instillation yielded virus.

Criteria for Reading Results—Positive symptoms and histopathology in both first and second passage animals fulfill the standard commonly accepted by workers in the field of experimental poliomyelitis, as indicating isolation of a virus. A considerable number of workers, however, especially in connection with the recent study of virus in the stools, report only one attempted passage. Positive results in the first passage

only would indicate the presence of the virus in the material tested but would not mean that virus had been recovered. Monkeys showing weakness or paralysis within 6 weeks after inoculation have been termed positive. In all questionable and in most of the clinically positive monkeys histopathologic confirmation has been obtained before a positive diagnosis was accepted.

FINDINGS AND DISCUSSION

Occurrence of symptomatic monkeys in both the first and second passages are included for comparison in Table I. Results of subsequent passages are reserved for a future report.

In the first passage, positive findings were obtained in 50 per cent of spinal cord medulla tissues, in 10 per cent of the olfactory bulbs, in 26 per cent of the colon contents, and in 50 per cent of the tonsil adenoid tissue tested from autopsies. 20 per cent of stools from active cases and 5 per cent of stools from carriers were positive.

Positive results in the second passages were obtained only with spinal cord medulla and tonsil adenoid tissue, from colon contents of autopsies, and from the stools of active cases and contacts. Other tissues and excreta as listed in Table I were tested without success.

1 Material from Autopsies—In the studies of autopsy material emphasis was placed on examination of cord medulla, brain cortex, olfactory bulbs, mesenteric lymph nodes, tonsil adenoid tissue, colon contents, and urine. Occasionally other tissues were tested as listed in Table I but all were negative.

Questionable results were procured in the case of one urine from an autopsy which has been omitted from the table. The monkey used for the test had previously been inoculated intranasally with stool from a patient had remained asymptomatic for 6 weeks and had been returned to stock. Owing to a shortage of stock animals it was promptly reinoculated intranasally with urine aseptically procured from a fatal case. 9 days later it developed marked weakness of the right leg and was sacrificed. The cord was only suggestively positive microscopically and was not infective for a second monkey. Since the results are questionable it is possible only to suggest that urine may at times contain virus.

Table II shows the results of attempts to recover virus from 22 autopsies from which both cord medulla and other tissues were tested. In this series, as judged by first passage results it will be seen that the cord medulla tissue was positive in 11 and negative in 11. In the autopsies in which the cord was positive, other materials, *i.e.*, tonsil adenoid tissue or colon contents were positive in 73 per cent while in the autopsies in which the cord was negative, all materials, with the exception of the one olfactory bulb also were negative.

In cases in which two materials were found to be positive from the same individual, the two strains of virus appear to produce similar symptomatology in monkeys.

In the main these findings are similar to those recently reported by Sabin and Ward (3). Their results do show motor cortex to have been positive while frontal and occipital cortex were negative. The portions of cortex tested in this series were taken from both frontal and motor areas. They report negative results with olfactory bulbs while one was positive in this series. These combined results indicate that occurrence of virus in olfactory bulbs, at least in sufficient amount to produce poliomyelitis in monkeys, is rare.

2 *Material from Cases—Cerebrospinal Fluid* As the work progressed 134 attempts were made to recover virus from spinal fluid of active cases. All were negative, though in one instance an injected animal exhibited question-

TABLE II
Autopsies from Which Multiple Materials Were Tested

Materials tested	Cord positive		Cord negative	
	Positive	Negative	Positive	Negative
Cord-medulla	11	0	0	11
Cerebral cortex	0	11	0	5
Olfactory bulb	0	6	1	3
Lymph node	0	9	0	5
Thymus	0	1	0	0
Hypophysis	0	3	0	2
Peyer's patch	0	0	0	1
Liver spleen	0	3	0	0
Bile	0	1	0	5
Colon contents	5	3	0	11
Urine	0	2	0	6
Tonsil adenoid	3	1	0	2

able symptoms. Sections from the cord, however, were negative so all attempts are reported as negative.

Nasal Washings Attempts also have been made to recover virus from nasal washings of patients. These were originated with the aid of Dr. Trask, Dr. Paul, and Dr. Webster, who were in Los Angeles during the early part of the poliomyelitis epidemic of 1934, at which time they succeeded in recovering virus from the nasal washings of one case (Paul, Trask, and Webster (4)). Of 139 attempts reported in this study, however, only 3 injected animals exhibited suggestive symptomatology and corroborative evidence was not presented by study of the tissues. Hence, all attempts are reported as negative. Such negative findings afford additional information with reference to the difficulty of detecting virus in nasal washings.

Stools Of the 53 stools obtained from active cases, 16 were examined in 1939 and 37 in 1940. The clinical type of disease of the patients, sex of the patients, and average age are shown in Table III. For comparison, the results of Trask,

Paul, and Vignec (5) have been included. Our figures indicate no significant differences between the percentage of individuals positive in the paralytic and non paralytic types nor between the percentages of males and females who were positive. Certain slight differences, however, are noted in the series of Trask, Paul, and Vignec.

Graphs 1 and 2 show an analysis of the stools from cases and of the colon contents from autopsies tested for virus. Graph 1 indicates the results of stool tests with reference to the time in the disease when they were collected and Graph 2 indicates the ages of the cases.

It will be observed from the graphs (1) that all isolations of virus from the stools or colon contents of patients and autopsies occurred within the first 3

TABLE III

Clinical Type of Disease and Sex of Patients Whose Stools Were Examined for Virus in First 4 Weeks of Disease

	Clinical type				Sex			
	Paalytic		Non paralytic		Male		Female	
	T P	L A †	T P	L A	T P	L A	T P	L A
Number of cases	38	27	15	26	32	35	21	18
Average age	9	16	11	13	9	14	10	20
Number positive	4	6	4	5	6	7	2	4
Per cent positive	10	22	27	19	18	20	10	22

Trask, Paul and Vignec

† Los Angeles

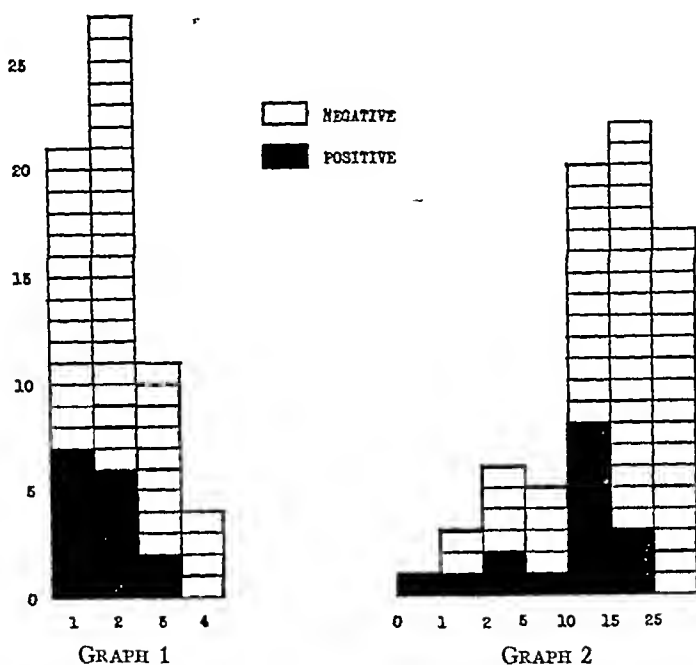
weeks after onset, (2) 40 per cent of the 35 patients under 15 years yielded virus while only 8 per cent of the 38 patients above 15 years yielded virus.

These results compare favorably with those of Trask, Paul, and Vignec (5), who procured 10 positives out of 63 cases tested. Howe and Bodian (6) report a much higher incidence of isolations. Sahm and Ward (3) also report a higher incidence of virus in stools of children under 5 years than in their age group 8 to 28. The facts that Howe and Bodian's cases were in the first decade of life and that most of the stools were examined in the first 2 weeks of the disease may be at least partially responsible for the high incidence they procured.

Comparison of Stool Isolations with Cord Medulla Isolations—While preparing the graphs of time and age distribution of cases whose stools were tested for virus it was thought that it might be of interest to prepare similar graphs which show the occurrence of virus in autopsy cords by weeks, after onset of the disease, and also the age of the autopsy cases. Graphs 3 and 4 portray this analysis and the following comments are of interest.

(1) All isolations of virus were from cases that died within 3 weeks after

onset (2) 55 per cent of attempted isolations in the 1st week and 56 per cent of attempted isolations in the 2nd week were positive, while only 14 per cent of the attempts in the 3rd week were positive (3) 71 per cent of 35 autopsies under 16 years yielded virus while 31 per cent of those 16 years and over yielded virus (4) 84 per cent of the 33 autopsies under 16 years of age and whose deaths occurred within 3 weeks after onset yielded virus This would



GRAPH 1 Recovery of virus from stools by weeks after onset

GRAPH 2 Recovery of virus from stools, showing ages of patients

appear to be a significant increase when compared with Table I which shows that only 50 per cent of all attempts were positive

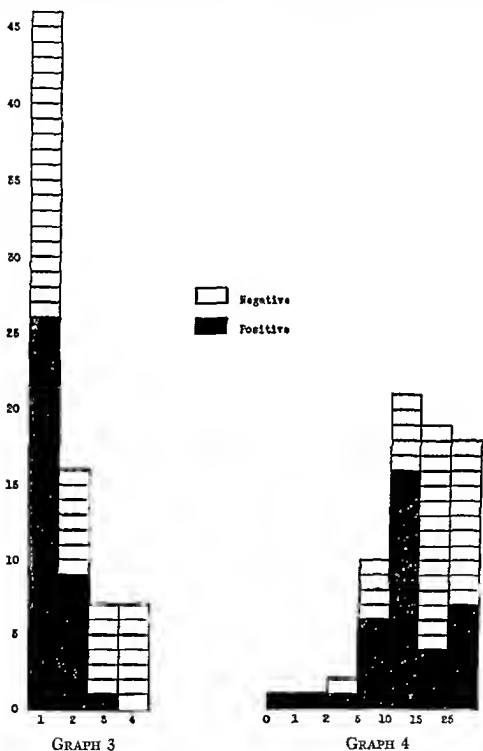
It will be observed that striking parallels exist between attempts to recover virus from stools and from cord-medulla tissue of autopsies in so far as age of patients and time in the course of disease when isolations were attempted. Two generalizations are indicated:

(1) A higher percentage of isolations have been obtained from children (below 16) than from adults

(2) The earlier in the course of the disease that attempts are made to isolate virus the greater the chance for success

Urine—Urine was collected from 7 cases who harbored virus in their stools. All were negative for virus.

3 *Material from Contacts*—Attempts were made to recover virus from stools of intimate contacts in families where multiple cases occurred or of cases which



GRAPH 3 Recovery of virus from autopsy cords by weeks after onset

GRAPH 4 Recovery of virus from autopsy cords showing ages of patients

suggested a possible contact history of special interest. Of 19 such contacts tested only one yielded virus.

The contact was a physician in the admitting room of this hospital whose wife developed paralytic poliomyelitis. He developed a very severe headache during the

first days of her illness This was without temperature and rare for him Otherwise he was entirely well and had no symptoms referable to poliomyelitis About 2 weeks later he noted a brief weakness in one foot while playing badminton We are inclined to attribute the headache to worry and fatigue and the foot weakness to sport, but these must be matters of opinion It is only possible to speculate as to whether the carrier transmitted the virus to the patient, whether he acquired the virus from the patient, or whether both patient and carrier acquired it from a common source

Table IV gives a tabulation of the results of Kramer, Gilliam, and Molner (7), of Trask, Paul, and Vignec (5), and of this summary in testing stools of contacts It is seen that in an institutional outbreak, Kramer *et al*, procured 20 per cent positive results of the individuals tested while Trask *et al*, procured negative results from varied sources In combining tests of all three surveys it is seen that 8 per cent of the 60 individuals examined harbored virus

TABLE IV
Stools from Contacts Tested for Virus

	Number positive	Number negative
Kramer <i>et al</i>	4	16
Trask <i>et al</i>	0	36
Kessel <i>et al</i>	1	18
Total	5	60
Per cent positive	8	

Repeat Stools—Second and third stools were collected from 1 to 3 months apart from 5 patients and one contact whose first stools examined contained virus 16 such stools were tested, only the first one from each patient being recorded in Table I All these repeat stools from patients were negative A positive stool, however, was obtained from the contact 2 months after the first positive but a stool examined 3 months after the first positive yielded no virus These observations afford corroborative epidemiologic evidence with reference to the dissemination of poliomyelitis virus in the stools of carriers

SUMMARY

- 1 Poliomyelitis virus has been recovered in monkeys from 50 per cent of spinal cords, 10 per cent of olfactory bulbs, 50 per cent of tonsil-adenoid tissue, and from 26 per cent of the colon contents of autopsies, from the stools of 20 per cent of patients, and of 5 per cent of the contacts examined in this series
- 2 Other materials as indicated in Table I were tested without success
- 3 In autopsies with positive cords, tonsil-adenoid tissues, or colon contents were positive in 73 per cent
- 4 22 per cent of stools from patients with paralysis yielded virus and 19 per cent of the stools from patients without paralysis yielded virus

5 20 per cent of the stools from males and 22 per cent of the stools from females yielded virus

6 40 per cent of 35 stools from patients under 16 years yielded virus while 8 per cent of 38 stools from patients above the age of 15 yielded virus

7 71 per cent of the cords of 35 autopsies under 16 years yielded virus while 31 per cent 16 years and over yielded virus

8 Repeat stools from 5 positive cases, 1 month after the first positive stool, were negative The stool of one contact was positive the 2nd month after first recovery but was negative the 3rd month

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THE BACTERICIDAL ACTION OF SYNTHETIC DETERGENTS

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In previous papers we have studied the action of synthetic detergents on the metabolism of bacteria (1, 2) By systematic investigation of the effects of some twenty cationic and anionic detergents on six Gram positive and six Gram negative microorganisms it was possible to derive certain generalizations between the type and structure of the detergents and their action on bacterial metabolism Some of these relationships could be inferred from other earlier studies,¹ many of which were on the bactericidal or bacteriostatic properties of a single detergent, but only by a detailed study of a variety of compounds could the general formulations be obtained In the present paper the systematic study is extended to germicidal properties of synthetic detergents The concentrations of detergents and the numbers of microorganisms are similar to those employed in the previous studies, thereby permitting a correlation between bactericidal properties and effects on bacterial metabolism

EXPERIMENTAL

The following bacteria were used in these studies Gram positive—*Staphylococcus aureus* *Staphylococcus albus*, and oral strains of lactobacillus and streptococcus (probably *Streptococcus salivarius*), Gram negative—*Proteus vulgaris* *Escherichia coli* and *Eberthella typhi* The bacteria were grown for 14 hours as previously described (2) centrifuged washed, and suspended in distilled water The suspensions were diluted to provide a final concentration of 10 billion microorganisms per cc

The detergents studied are listed in Table I They are classified as cationic or anionic according to the location of the long-chain, hydrophobic group in the cationic

¹ The reader is referred to the paper by Baker Harrison, and Miller (2) for a relatively complete bibliography of the biological applications of synthetic detergents In addition the recent papers by Kuhn and coworkers (3-8) should be consulted

² This value necessarily includes some non viable bacteria since the number of cells was estimated by means of a Petroff Hausser counting chamber

or anionic portion of the molecule. The compounds employed in these studies were chosen to provide as much variety in chemical structure as possible in order to obtain additional information concerning the relationship between chemical structure and biological activity. They were dissolved in distilled water and neutralized to pH 7. In order to obtain results directly comparable with the metabolism

TABLE I
Composition of the Detergents

Type	Trade name	Structure
Cationic	Zephuran	Mixture of alkyl dimethyl benzyl ammonium chlorides (alkyl = C ₈ to C ₁₈)
	Phemerol	Alkyl dimethyl benzyl ammonium chloride (alkyl = para-tertiary-octyl phenyl diethoxy)
	Retarder LA	Stearyl trimethyl ammonium bromide
	Emulsol-605	$C_{11}H_{23}-COO-C_2H_4-NH-CO-CH-N(CH_3)_3Cl$
	Catol	$C_{11}H_{23}-COO-C_2H_4-NH-CO-CH_2-N(C_2H_5)_3Cl$
	Emulsol-607	$C_{11}H_{23}-COO-C_2H_4-NH-CO-CH-N \begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{Cl} \end{array}$
	Emulsol 660 B	Lauryl pyridinium iodide
	Damol	N N N' N'-tetramethyl-N-N'-didodecyl-beta-hydroxy-propylene diammonium bromide
Anionic	Emulsol-609	Lauryl ester of alpha amino isobutyric acid hydrochloride
	Cetyl sulfate	Sodium cetyl sulfate (C ₁₆)
	Sodium myristyl sulfate	Sodium myristyl sulfate (C ₁₄)
	Duponol LS	Sodium oleyl sulfate (C ₁₈)
	Tergitol-8	Sodium alkyl sulfate (alkyl derived from 2-ethyl hexanol-1) (C ₈)
	Triton W-30	Sodium salt of alkyl phenoxy ethyl sulfonate
	Igepon T	Alkyl-CO-N(CH ₃)-C ₂ H ₄ -SO ₃ Na
	Tergitol-7	Sodium alkyl sulfate (alkyl derived from 3,9 diethyl tridecanol-6) (C ₁₇)
	Tergitol-4	Sodium alkyl sulfate (alkyl derived from 2, methyl-7, ethyl undecanol-4) (C ₁₁)

ously reported, the experiments were set up in the following manner: A detergent solution was added to 18 cc of M/15 phosphate buffer (pH 7), which glucose to produce a final concentration of 0.02 M. The tubes were placed in a water bath until the temperature of the bath was reached. The suspension was added. After intervals of 10 minutes and 90 minutes, the tubes were withdrawn and added into 5 cc of veal infusion broth.³ A

with 10

streptococci, the veal infusion broth con-

second transfer of 0.1 cc. was made from the first tube of veal infusion broth into another tube containing 5 cc. of culture medium to eliminate bacteriostatic action.⁴ The tubes were incubated at 37°C. and examined for growth after 24 and 48 hours (or 72 hours for slowly growing organisms like lactobacilli and streptococci). It should be noted that the number of bacteria employed in our tests is probably at least ten times the number subjected to the germicides in the standard phenol coefficient determination. Moreover in the latter technique only a loopful of bacteria is carried over from the medication tube into the broth culture tube whereas in our tests 0.1 cc. transfers were employed routinely.

RESULTS

Results obtained after exposure of the various organisms for 10 and 90 minutes to the detergents at concentrations of 1:1000, 1:3000, 1:6000, and 1:30,000 and at pH 7 are listed in Tables II to VIII. The data given were obtained by noting the presence or absence of growth in the *second* transfer tube or broth medium, these results therefore represent *bactericidal* action. A plus or minus sign is used to indicate the presence or absence of growth. Each plus or minus sign represents the results of at least two separate experiments. Some variations were observed. When the results were inconclusive in three or more experiments this fact is indicated by a question mark.

Gram Positive Microorganisms—The results obtained in the tests with streptococcus, lactobacillus, *Staphylococcus aureus*, and *Staphylococcus albus* are given in Tables II to V. It is apparent that the cationic detergents are much more effective than the anionic detergents. All the cationic detergents except Emulsol-609 gave complete sterilization in 10 minutes against these four Gram positive organisms at the 1:1000 and 1:3000 dilutions. At 1:6000 dilution most of the cationic detergents were still able to give complete killing in 10 minutes. At 1:30,000 concentration there was a sharp diminution in complete killing effects. Only Zepbiran and Phemerol were capable of killing in 10 minutes at this concentration, and the effects of these compounds were not consistent for the four organisms. In the 90 minute period of exposure both of these compounds consistently killed the four species of Gram positive organisms. Even with the longer period of exposure, only three of the other compounds gave complete sterilization at 1:30,000, i.e., Retarder LA with lactobacillus and *Staphylococcus aureus*, Damol with lactobacillus and Emulsol 609 with *Staphylococcus albus*.

⁴ The biggest concentration of detergent in the second transfer tubes prepared in this manner is 1:2,500,000, a 50-fold dilution is made from the medication tube to the first transfer tube and again from the first to the second transfer tube thus giving an overall dilution of 2500 times that in the original medication tube. Experiments which we have performed demonstrate that a concentration of 1:2,500,000 is definitely beyond the bacteriostatic range of the compounds studied, in almost all instances the bacteriostatic concentration was considerably below 1:1,000,000.

Of the anionic detergents studied, it is apparent that Tergitol-7 is the most active, and it is the only one whose activity compares reasonably well with the cationic detergents against streptococcus and lactobacillus. Against both

TABLE VI
Growth of Proteus vulgaris after Exposure to Detergents

Type	Detergent	Time of exposure							
		10 min				90 min			
		Concentration of detergent							
		1 1000	1 3000	1 6000	1 30 000	1 1000	1 3000	1 6000	1 30,000
Cationic	Zephuran	—	—	?	+	—	—	—	+
	Phemerol	—	—	+	+	—	—	—	+
	Retarder LA	+	+	+	+	—	—	+	+
	Emulsol-605	—	—	+	+	—	—	—	+
	Catol	—	—	+	+	—	—	+	+
	Emulsol-607	—	+	+	+	—	—	+	+
	Emulsol-660 B	—	?	+	+	—	—	+	+
	Damol	?	?	+	+	—	—	+	+
	Emulsol 609	+	+	+	+	+	+	+	+

TABLE VII
Growth of Escherichia coli after Exposure to Detergents

Type	Detergent	Time of exposure							
		10 min				90 min			
		Concentration of Detergent							
		1 1000	1 3000	1 6000	1 30 000	1 1000	1 3000	1 6000	1 30 000
Cationic	Zephuran	—	—	—	+	—	—	—	+
	Phemerol	—	—	—	+	—	?	—	+
	Retarder LA	—	+	+	+	—	—	?	+
	Emulsol 605	—	—	—	+	—	—	—	+
	Catol	—	—	—	+	—	—	—	+
	Emulsol 607	—	—	—	+	—	—	—	+
	Emulsol 660 B	—	?	+	+	—	—	—	+
	Damol	—	—	—	+	—	—	—	+
	Emulsol 609	+	+	+	+	—	?	+	+

strains of staphylococcus Tergitol-7 is the only anionic detergent which shows some effect, but its activity is decidedly inferior to the cationic compounds against the staphylococci.

Gram-Negative Microorganisms—The results of tests upon three Gram-negative organisms are given in Tables VI to VIII. Only the results with the cationic detergents are tabulated because *none of the anionic detergents was*

effective against any of the three species of Gram negative organisms even at the highest concentration studied⁵

The cationic detergents are somewhat less effective against these Gram negative organisms than against the Gram positive ones. The action on *E coli* and *Eb typhi* was greater than upon *Proteus vulgaris*. A few of the compounds, e g, Emulsol 609 and Retarder LA, had very little effect on *P vulgaris*. No compound killed any of the species at 1:30 000 dilution even after 90 minutes exposure, at 1:6000 dilution and 90 minutes incubation, Zephiran, Emulsol-605, and Phemerol were the only ones effective against all three

TABLE VIII
Growth of *Eberthella typhi* after Exposure to Detergents

Type	Detergent	Time of exposure							
		10 min				90 min			
		Concentration of detergent							
		1 1000	1 3000	1 6000	1 30 000	1 1000	1 3000	1 6000	1 30 000
Cationic	Zephiran	-	-	-	+	-	-	-	+
	Phemerol	-	-	+	+	-	-	-	+
	Retarder LA	-	-	+	+	-	-	-	+
	Emulsol 605	-	-	-	+	-	-	-	+
	Catol	-	-	-	+	-	-	-	+
	Emulsol-607	-	-	-	+	-	-	+	+
	Emulsol-660 B	-	+	+	+	-	-	-	+
	Damol	-	-	-	+	-	-	-	+
	Emulsol-609	+	+	+	+	-	-	-	+

species. Several of the others were effective against *E coli* and *Eb typhi* but not against *P vulgaris* at 1:6000 with the 10 minute period of exposure.

DISCUSSION

Our previous experiments showed that all the cationic detergents observed inhibit the *respiration* or *acid production* of the Gram positive and negative

⁵Dr R J Dubos has recently reported (9) a difference in activity between the anionic and cationic detergents toward the Gram positive and Gram negative species when tested with detergents and wetting agents and has suggested that if this difference exemplifies a general law 'it may serve as a useful tool for the study of some points of bacterial structure and give important clues concerning the factors which render Gram negative organisms resistant to so many agents which are toxic to the Gram positive

It would appear from the good agreement between the results of Dubos *et al* and the work reported in our own paper that the 'general law' governing the action of the detergents has been reasonably well established

microorganisms included in the present study⁶ The inhibition was always complete at a concentration of 1 3000, and usually at 1 30,000 At higher dilutions, *e g* , 1 60,000, these complete inhibitions have been observed less frequently (unpublished experiments) When complete inhibition is obtained with the detergents the action is very rapid, usually respiration and glycolysis have ceased almost entirely at the time of the first manometric reading (15 minutes after the addition of the detergent) The present studies on the bactericidal action of the detergents have been conducted in precisely the same manner as those on bacterial metabolism with the exception that in some instances fewer organisms have been employed On the average, however, the results are directly comparable⁷ With the Gram-positive organisms there appears to be a fairly good correlation between the bactericidal action and the inhibition of bacterial metabolism In most instances, in the 10 minute period of exposure, the cationic detergents kill all the organisms at a concentration between 1 6000 and 1 30,000 With the Gram-negative organisms the correlation is less marked, killing was not observed at 1 30,000 even though this concentration is sufficient to exert a very marked inhibition of the metabolism of many Gram-negative species⁸

It is true that there are instances in our results where no correlation at all can be observed between effect on metabolism and germicidal action of certain compounds For example, Retarder LA was unable to kill *Proteus vulgaris* in 10 minutes at 1 1000 concentration, yet it inhibits the respiration of this organ-

⁶ In our previous study we did not report results obtained with the following six compounds included in the present study Phemerol, Catol, Emulsol-607, Emulsol-609, and Tergitol- 8 and 4 The structural formulas of these compounds are indicated in Table I It may be noted briefly from unpublished experiments that the first four compounds, all cationic, are effective inhibitors of the metabolism of Gram-positive and Gram-negative microorganisms at a concentration of 1 3000, and some are equally effective at 1 30,000 Tergitol-8 is relatively inactive, and Tergitol-4 falls in the same range of activity as cetyl sulfate, showing considerable variations in its inhibitory action

⁷ Unfortunately, determination of killing power, based on cell viability after transfer to broth provides indications only of an all-or-none reaction As many as 90 per cent of the organisms may have been killed after 10 or 90 minutes exposure to the detergent, yet given a long enough time, the growth will be as great as in the control tube Therefore, data obtained in this manner cannot be as quantitatively exact as those obtained in the metabolism studies

⁸ As shown in the accompanying paper the antibacterial effects of both cationic and anionic detergents can be inhibited by appropriate concentrations of phospholipids It is conceivable that the complete absence of bactericidal action of anionic detergents and the diminished activity of cationic detergents on Gram-negative organisms, may be related to differences in content, type, or cellular arrangement of phospholipids in Gram-negative as compared with Gram-positive species

ism 85 per cent at concentrations of 1:3000 and 1:30,000. Similarly, the non quaternary nitrogen type of cationic detergent, Emulsol 609, which had been shown previously to inhibit the respiration of *Proteus vulgaris* very markedly, did not exhibit any effective germicidal action against this organism. In general, however, the correlation of effectiveness against bacterial metabolism and the bactericidal properties in the detergent group of compounds seems to be good enough to permit the assumption that the general relations found previously can be applied to the bactericidal effects with some degree of validity. This would mean that as a class the cationic detergents should be more effective germicides at neutrality and slightly alkaline pH than on the acid side, and that the anionic detergents will behave in the opposite manner.⁹ It can be predicted that the bactericidal activity of both types of detergents will be dependent on optimal lengths of the alkyl chain in the molecule.

Certain relationships appear to exist between chemical configuration of the detergents and their bactericidal properties. Zephiran and Phemerol were somewhat superior to all the other compounds tested. The former is a mixture of alkyl dimethyl benzyl ammonium chlorides in which the alkyl groups vary from C₈ to C₁₃, and the latter compound is an alkyl dimethyl benzyl ammonium chloride in which the alkyl group is para tertiary octyl pbenyl-diethoxy. The alkyl portions of these two compounds differ markedly, yet they resemble each other in possessing a benzyl group attached to the quaternary nitrogen. The other seven cationic detergents studied do not contain a benzyl group. It seems reasonable to assume that the introduction of the benzyl group in Zephiran and Phemerol has enhanced their bactericidal properties.

The substitution of ethyl groups for methyl groups on the quaternary nitrogen appears to make little difference since Catol (alkyl triethyl ammonium chloride) and Emulsol 605 (alkyl trimethyl ammonium chloride) were equally effective. The presence of a quaternary nitrogen in a ring structure, e.g., pyridinium nitrogen in lauryl pyridinium iodide (Emulsol 660 B) and alkyl pyridinium chloride (Emulsol 607), does not increase bactericidal properties above those exhibited by acyclic quaternary nitrogen compounds.

In the group of *anionic detergents* molecular configuration plays an important rôle. It was shown that Tergitol 7, and to a lesser extent Tergitol-4, exerted considerable bactericidal properties. Both these compounds are derived from branched chain, secondary alcohols and therefore differ markedly from the other anionic detergents which are derived from straight-chain, primary alcohols. The alkyl group in Tergitol 7, for example, is derived from 3, 9 diethyl tridecanol-6. It is interesting to note the great differences exhibited by Tergitol 7 within the class of Gram positive organisms. Against lacto-

⁹ Gershenfeld and Perlstein (10) have shown recently that the anionic detergent Aerosol OT, exerts a considerable bactericidal effect against staphylococci at acid pH values.

bacillus it has an unusually good germicidal action, especially when one considers that the pH employed is not the optimum for the anionic type of detergents. Against the streptococcus, it is somewhat less effective but still quite powerful, whereas against staphylococcus it showed only slight killing effect. At lower pH's it will undoubtedly be found to exert a quite marked activity against even the staphylococcus. However, it is interesting to find in these studies the same effect that has often been observed with ordinary soaps, *i e*, diminished bactericidal activity toward the staphylococcus as compared with other Gram-positive organisms. It is of interest to note, however, that in our previous study we found that Tergitol-7 inhibited the metabolism of the staphylococcus as completely as that of other Gram-positive microorganisms.

SUMMARY

1 The bactericidal action of a number of anionic and cationic synthetic detergents on four Gram-positive and three Gram-negative bacteria has been investigated.

2 Cationic detergents, as a group, were found to exhibit marked bactericidal effects on Gram-positive microorganisms and somewhat less pronounced action on Gram-negative organisms.

3 The anionic detergents were germicidal only against the Gram-positive organisms, and they were considerably less effective than the cationic compounds. Of the anionic detergents, the most active one was an alkyl sulfate derived from a branched-chain, secondary alcohol.

4 Correlations between bactericidal action and inhibition of bacterial metabolism, and also between bactericidal action and chemical structure of the detergents are discussed.

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INHIBITION BY PHOSPHOLIPIDS OF THE ACTION OF SYNTHETIC DETERGENTS ON BACTERIA

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Certain of the synthetic detergents exert a marked inhibitory effect on the metabolism (1) and viability (2) of bacteria. The germicidal action of these compounds and other biological effects have been studied by numerous investigators.¹

Various explanations have been proposed for the action of these compounds on bacteria, but none of these has been proved experimentally. Kuhn and Bielig (3) have suggested recently that germicidal concentrations of some detergents correspond rather closely with the concentrations necessary to effect denaturation of proteins. However, the relative activity of the detergents in protein denaturation as reported by Anson (4) does not correspond with the effects which we have observed on bacterial metabolism and viability. Furthermore, anionic detergents which denature proteins readily are highly selective in their action on bacteria. These compounds inhibit only Gram positive microorganisms.¹ Obviously one or more factors besides denaturation of proteins must influence the activity of the anionic detergents. Possibly in the organized cell there is the additional factor of interaction between the detergents and the lipid constituents of the cellular membrane.

The rôle of lipoids in the membrane of cells has received considerable attention. It has been suggested that the protoplasmic membrane consists of a continuous lipid structure (5), a lipoprotein mosaic (6) or a layer of lipid molecules between adsorbed protein layers (7-9). The exact nature of the lipid constituent is not known. However, Bungenberg de Jong and Bonner (10) have suggested as a working hypothesis that 'the special properties of the protoplasmic membrane depend upon one or more layers of oriented phosphatide ions.'

A number of studies on the action of phospholipids on cells have been made. These compounds have been reported to weaken the antiseptic action of mercuric chloride, phenol, and salvarsan on antbrax bacilli (11) and to inhibit the action of staphylococcus bacteriophage (12, 13) and of various bacterial lysins and toxins (14-18). A

¹ See reference 1 for bibliography.

number of workers (19-22) believe that cytolysis and hemolysis by saponins are reduced by phosphatides

Since the detergents are known to be highly surface-active, and their initial effect, in all probability, is to disorganize the cell membrane, it was of interest to determine the influence of added phospholipids on the inhibitory and germicidal action of detergents. As shown later, it appears that certain surface-active compounds such as the phospholipids can modify the activity of detergents very markedly

Methods

Bacterial Metabolism—All experiments were conducted in Warburg manometers as previously described (1). The bacterial suspensions were prepared from bacteria grown on veal infusion agar. *Lactobacillus* was grown in 1 per cent glucose-meat infusion broth. Respiration and glycolysis experiments were conducted in phosphate and bicarbonate buffers, respectively, containing glucose. The phospholipids were added directly to the bacterial suspension in the vessel, the detergents were pipetted into the side bulb and added at the start of the experiment unless otherwise specified.

Bactericidal Action—The bactericidal potency of the detergents was determined under precisely the same experimental conditions as were employed in the studies on bacterial metabolism. This makes possible direct comparison between these two phenomena. The bacterial suspension was diluted so as to contain 10 billion cells per cc, 1.0 cc of this suspension was added to 1.8 cc of phosphate buffer, (pH 7.0), and 0.1 cc of phospholipid solution. Controls were made up in the same manner without phospholipid. The tubes were placed in a water bath at 38°, 0.2 cc of detergent solution, sufficient to provide the desired final concentration, was then added. After 1 hour, 0.1 cc of this mixture was pipetted into 5 cc of veal infusion broth (containing 0.2 per cent glucose for *lactobacillus* experiments). A second transfer of 0.1 cc was made from the first tube of veal infusion broth to eliminate the possibility of bacteriostatic action. The tubes were examined for growth after 48 or 72 hours. Only data on the second subculture tubes are recorded.

Detergents—The detergents used have been described in previous publications (1, 2) and are referred to here as *cationic* or *anionic*, depending on whether the long-chain, hydrophobic group is in the cation or anion. A few experiments were performed with an unionized detergent. The compounds were dissolved in water and neutralized to pH 7.0.

Phospholipids—Phospholipids from various sources were used for these experiments. We wish to express our indebtedness to the following individuals and companies for supplying purified preparations of these compounds: soy-bean lecithin, (Emulsol Corporation), soy-bean lecithin and cephalin, the latter purified through the cadmium salt (Dr Percy Julian, Glidden Company), beef heart lecithin and egg lecithin from Dr Mary C Pangborn, as prepared by her improved technique (23), brain cephalin and sphingomyelin from Dr H N Christiansen, described in (24), brain cephalin (phosphatidyl serine) from Dr Jordi Folch (25). Aqueous suspensions of the phospholipids were prepared and diluted as desired.

RESULTS ON BACTERIAL METABOLISM

Effect of Soy Bean Lecithin

Most of our first experiments were performed with a purified preparation of soy bean lecithin (obtained from Emulsol Corporation) which also contained some cephalin. Upon addition of this lecithin to a suspension of bacteria it was found that the usual inhibition of bacterial metabolism by detergents did not occur. To produce inhibition in the presence of lecithin considerably higher concentrations of detergents were required. These results were obtained with both Gram positive and Gram negative microorganisms, and with cationic and anionic detergents (Tables I and II). Thus, in Experiment 1 of Table I on *aerobic acid production* by *Lactobacillus*, 0.1 mg Zephiran inhibited 100 per cent, on the addition of 3.0 mg lecithin, no inhibition was observed. Similarly, 0.1 mg Emulsol 660 B and 0.1 mg Emulsol-609 inhibited 43 and 91 per cent, respectively, in the presence of lecithin, there was no inhibition. As shown also in Table I, the same type of results was obtained with *Micrococcus tetragenus* (Experiment 2). The inhibitory effect of Zephiran on the *anaerobic acid production* of *Staphylococcus aureus* was also prevented by lecithin (Experiment 3).

Experiments 4 and 5 demonstrate that lecithin prevents the inhibitory action of cationic detergents on the *respiration* of the Gram positive organisms, *Staphylococcus aureus* and *Sarcina lutea*. Experiments 6 and 7 demonstrate a similar action on the respiration of the Gram negative microorganisms, *Proteus vulgaris* and *Escherichia coli*.

The three cationic detergents were chosen to provide a wide variation in chemical structure. Zephiran (alkyl dimethyl benzyl ammonium chloride) represents the *acyclic* quaternary nitrogen type, Emulsol-660 B (lauryl pyridinium iodide), the *cyclic* quaternary nitrogen type, and Emulsol-609 (lauryl ester of alpha amino isobutyric acid hydrochloride), the non quaternary nitrogen type of cationic detergent. All of these detergents were influenced to the same degree by lecithin.

The experiments in Table II show that lecithin can also prevent the inhibitory action of the anionic detergents. Only the Gram positive microorganisms, *Staphylococcus aureus* and *Lactobacillus*, are included in this table since previous studies demonstrated that the anionic detergents inhibit the metabolism of Gram positive organisms only. It can be seen from Table II that the inhibitory action of the anionic detergents, Tergitol 7, cetyl sulfate, Duponol LS, and Triton W 30, is largely prevented by 3.0 mg of lecithin. When the concentration of Tergitol 7 was increased to 0.5 mg, this concentration of lecithin was sufficient to protect *Staphylococcus aureus*, but not *Lactobacillus*.

It can be seen from Tables I and II (and subsequent tables) that in a number

of instances, *stimulation* of acid production or respiration occurred in control experiments with lecithin. In general, this is a genuine stimulation. For example, in the experiments on acid production by lactobacillus, analysis

TABLE I

Effect of Soy-Bean Lecithin on Inhibition of Bacterial Metabolism by Cationic Detergents

Experiment	Bacteria	Detergent	Concentration of detergent	Concentration of lecithin	Inhibition*
			mg /3 cc	mg /3 cc	per cent
1	Lactobacillus‡	Zephuran	0	3 0	+9
			0 1	0	-100
			0 1	3 0	0
		Emulsol-660 B	0	3 0	+5
			0 1	0	-43
			0 1	3 0	+5
		Emulsol-609	0	3 0	+5
			0 1	0	-91
			0 1	3 0	0
2	Micrococcus tetragenus‡	Zephuran	0	3 0	+3
			0 1	0	-89
			0 1	3 0	+5
		Emulsol 660 B	0	3 0	+3
			0 1	0	-83
			0 1	3 0	-2
3	Staphylococcus aureus§	Zephuran	0	3 0	+36
			0 1	0	-85
			0 1	3 0	+8
4	Staphylococcus aureus	Zephuran	0	3 0	+45
			0 05	0	-81
			0 05	3 0	+40
			0 05	0 3	+32
			0 05	0 1	-30
			0 05	0 05	-48
		Emulsol 660 B	0	3 0	+48
			0 1	0	-86
			0 1	3 0	+46
		Emulsol 609	0	3 0	+48
			0 2	0	-85
			0 2	3 0	+41

TABLE I—Concluded

Experi- ment	Bacteria	Detergent	Concentra- tion of detergent	Concentra- tion of lecithin	Inhibition
5	<i>Sarcina lutea</i>	Zephuran	mg /3 cc	mg /3 cc	per cent
			0	3 0	+65
			0 1	0	-85
			0 1	3 0	+112
		Emulsol-660 B	0	3 0	+65
			0 1	0	-71
			0 1	3 0	+114
		Emulsol-609	0	3 0	+65
			0 2	0	-85
			0 2	3 0	+119
6	<i>Proteus vulgaris</i>	Zephuran	0	0 3	+14
			0 05	0	-41
			0 05	0 3	+18
7	<i>Escherichia coli</i>	Zephuran	0	3 0	+13
			0 05	0	-93
			0 05	3 0	+13
		Emulsol-660 B	0	3 0	+13
			0 1	0	-88
			0 1	3 0	-6
		Emulsol 609	0	3 0	+13
			0 2	0	-84
			0 2	3 0	-2

* Minus signs represent inhibition plus signs represent stimulation

† Aerobic glycolysis

‡ Anaerobic glycolysis

|| Respiration

showed that the increase in acid, indicated by manometric readings, was accounted for by increased glucose utilization and increased lactic acid production, (the latter estimated by the method of Miller and Muntz (26)), no acid was produced from lecithin by bacterial action. Although this effect has been observed with highly purified preparations it is impossible at present to say with certainty whether the stimulation is caused by the phospholipid or traces of catalytic impurity. In a few cases in which glucose estimations were made in respiration experiments however, a "sparing" action was observed in the presence of lecithin, i.e., less glucose was used than in the control vessel. Presumably oxidation of lecithin accounted for the extra oxygen uptake.

Other Phospholipids

Essentially the same results as those presented in Tables I and II were obtained with other phospholipids studied. The most active compounds were

TABLE II

Effect of Soy-Bean Lecithin on Inhibition of Bacterial Metabolism by Anionic Detergents

Bacteria	Detergent	Concentration of detergent	Concentration of lecithin	Inhibition*
		mg /3 cc	mg /3 cc	per cent
<i>Staphylococcus aureus</i> †	Tergitol-7	0	3 0	+26
		0 3	0	-90
		0 3	3 0	-5
		0 5	0	-88
		0 5	3 0	+25
Lactobacillus§	Tergitol-7	0	3 0	+79
		0 2	0	-94
		0 3	0	-91
		0 5	0	-96
		0 2	3 0	+63
		0 3	3 0	+27
		0 5	3 0	-81
	Cetyl sulfate	0	3 0	+60
		0 1	0	-94
		0 2	0	-94
		0 1	3 0	+42
		0 2	3 0	+48
	Duponol LS	0	3 0	+60
		0 1	0	-36
		0 2	0	-79
		0 1	3 0	+52
		0 2	3 0	+46
	Triton W-30	0	3 0	+60
		0 2	0	-84
		0 2	3 0	+38

* Minus signs represent inhibition, plus signs represent stimulation

† Respiration

§ Anaerobic glycolysis

cephalins and soy-bean lecithin. Some typical results with Zephiran are reproduced in Table III. Similar results have been obtained with other detergents. The data in this table indicate that the phospholipids are effective at very low concentrations. Thus, in several instances, 0.15 mg of cephalin or lecithin (approximately $M/20,000$) was sufficient to reduce considerably the

inhibitory action of 0.05 mg of Zephiran. In some cases as little as 0.05 mg phospholipid reduced the inhibition.

TABLE III
Effect of Various Phospholipids on Inhibition by Zephiran

Bacteria	Phospholipid	Inhibition by 0.05 mg Zephiran Concentration of phospholipid mg./3 cc				
		0.0	0.05	0.15	0.3	0.5
		per cent	per cent	per cent	per cent	per cent
<i>Staphylococcus aureus</i> †	Beef lecithin	-87	-54	-19	-12	+8
	Egg lecithin	-87	-77	-79	-71	-54
	Soy lecithin	-87	-55	-26	-12	-2
	Soy cephalin	-87	-75	-48	-15	-5
	Sphingomyelin	-87	-75	-70	-42	-36
	Brain cephalin§	-78	-45	-21	+17	+29
<i>Escherichia coli</i> ‡	Beef lecithin	-81	-72	-57	-50	-23
	Egg lecithin	-81	-76	-69	-58	-33
	Soy lecithin	-81	-71	-41	-8	+3
	Soy cephalin	-81	-70	-29	-6	-1
	Sphingomyelin	-81	-73	-47	-26	-5
	Brain cephalin§	-89	-86	-36	-4	-6
<i>Lactobacillus</i>	Beef lecithin	-85	-61	-45	-41	-31
	Egg lecithin	-85	-63	-47	-37	-35
	Soy lecithin	-85	-50	-30	-3	-2
	Soy cephalin	-85	-58	-41	-21	-1
	Sphingomyelin	-85	-48	-44	-33	-32
	Brain cephalin**	-79	-48	-45	-19	-11
	Brain cephalin§	-89	-38	-26	-10	+1

* Minus signs represent inhibition plus signs represent stimulation

† Respiration

‡ Phosphatidyl serine.

§ Aerobic glycolysis

** Cephalin prepared by Dr H N Christensen

Time Relationship and Period of Exposure

Previous experiments have shown that the detergents act very rapidly and that their inhibitory effect on bacterial metabolism is completed usually within 5 minutes. We have found in the present study that if bacteria are exposed to the detergent *first*, subsequent addition of phospholipid cannot prevent the inhibitory action. The phospholipid must be added *before or simultaneously* with the detergent. Thus, in an experiment with *Staphylococcus aureus*, 0.05 mg of Zephiran produced an inhibition of 81 per cent, 3.0 mg of soy bean lecithin, added 15 minutes later, had no effect on the inhibition. On the other

hand, when either 0.1 mg or 0.3 mg of lecithin was added *before* the detergent, the phospholipid prevented the inhibition by Zephiran.

If bacteria are exposed to a solution containing lecithin for several minutes, and then *removed* from the lecithin solution and washed, they continue to be resistant to concentrations of detergents which are normally inhibitory. Protection induced in this manner is dependent on the concentration of both the phospholipid and detergent. A typical experiment was carried out as follows: a suspension of *E. coli* (1.0 cc) was treated with 3 mg of soy-bean lecithin, after 5 minutes the cells were centrifuged, washed, recentrifuged, and suspended in their original volume. A similar suspension, treated in the same manner but without lecithin, served as a control. Subsequent experiments on respiration in the presence of glucose plus 0.05 mg Zephiran gave the following results: control, 88 per cent inhibition, lecithin-treated, 2 per cent inhibition. Similar results were obtained with *Staphylococcus aureus*, *Proteus vulgaris*, and lactobacillus.

Addition of Lecithin to Culture Medium

Several organisms were grown in the presence of soy-bean lecithin (1 mg/cc) to determine if this would affect subsequent sensitivity to the detergents. The results obtained with *Staphylococcus aureus*, *E. coli*, and lactobacillus are indicated in Table IV. It is apparent that there was no change in the sensitivity of *Staphylococcus aureus* and *E. coli*. The inhibitions with Zephiran at various concentrations were almost precisely the same as those of the controls (grown normally, in the absence of lecithin). On the other hand, two experiments with lactobacillus indicated that considerably higher concentrations of Zephiran or Tergitol-7 were necessary to inhibit cells grown in the presence of lecithin. Possibly, further experiments with *Staphylococcus aureus* and *E. coli* at different concentrations of phospholipid might lead to the same result obtained with lactobacillus.

Effect of Phospholipids on the Bactericidal Action of Detergents

A few experiments were performed to determine whether the phospholipids could prevent the *bactericidal* action of the detergents. The results obtained are presented in Table V. Bactericidal action (absence of growth) is indicated by a minus sign. The values recorded were obtained by examination of the second subculture tubes after 48 or 72 hours incubation. It is apparent that a considerably higher concentration of detergent is required to kill the microorganisms in the presence of phospholipid than in its absence, and that the phospholipid is effective in a relatively low concentration.

With *Staphylococcus aureus* and *E. coli*, the bactericidal action of 0.3 and 0.5 mg of Zephiran and Phemerol was prevented by 3.0 mg of lecithin. With lactobacillus the following results were obtained: 3 mg of lecithin prevented

the bactericidal action of 0.1 mg of Zephiran or Phemerol (1/30,000). Moreover, 1.0 mg, but not 0.5 mg, of lecithin protected against 0.1 mg of Zephiran. 3.0 mg of lecithin was not sufficient to protect against 0.5 mg of Zephiran, but was effective against 0.3 and 0.5 mg of Tergitol 7 (but not against 1.0 mg of

TABLE IV
Effect of Inclusion of Lecithin in Culture Medium

Bacteria	Culture medium	Concentration of detergent		Inhibition of metabolism by detergents	
		Zephiran	Tergitol 7	Cultured in absence of lecithin	Cultured in presence of lecithin
		mg / 3 cc	mg / 3 cc	per cent	per cent
<i>Staphylococcus aureus</i> †	Broth	0.05		70	67
		0.10		84	85
		0.20		86	87
		0.30		87	82
<i>E. coli</i> ‡	Agar	0.05		80	80
		0.10		89	91
		0.20		92	91
<i>Lactobacillus</i> §	Broth	0.10		82	8
		0.15		91	15
		0.20		92	13
		0.30		92	42
<i>Lactobacillus</i> §	Broth	0.05		79	9
		0.10		92	11
		0.20		91	32
		0.30		94	83
			0.10	90	9
			0.20	96	17
			0.30	95	96

* 1.0 mg / cc

† Respiration

§ Aerobic glycolysis

Tergitol 7) It is apparent from these results that just as in the experiments on bacterial metabolism, there is a close relationship between the concentration of bactericidal agent and the effective concentration of phospholipid.

Effect of Phospholipids on Inhibition by Other Germicidal Compounds

The effect of lecithin on the inhibition of metabolism by mercuric chloride and by two organic mercury compounds, Merthiolate and Metaphen, has been studied. The results are summarized in Table VI. In no instance did 3 mg

of lecithin adequately protect against 0.05 or 0.1 mg of these compounds (in 3 cc volume). However, when the concentration of mercuric chloride was very low (*viz.* 0.01 mg) 3.0 mg of lecithin prevented the inhibition (Experi-

TABLE V
*Effect of Phospholipids on Bactericidal Action of Detergents**

Bacteria	Detergent	Concentration of detergent	Concentration of lecithin			
			0	0.5	1.0	3.0
		mg /3 cc	mg /3 cc	mg /3 cc	mg /3 cc	mg /3 cc
<i>Staphylococcus aureus</i>	Zephiran	0.5	—			+
		0.3	—			+
		0.1	—			+
	Phemerol	0.5	—			+
		0.3	—			+
<i>E. coli</i>	Zephiran	0.5	—			+
		0.3	—			+
	Phemerol	0.5	—			+
		0.3	—			+
Lactobacillus	Zephiran	0.1	—			+
	Phemerol	0.1	—			+
Lactobacillus	Zephiran	0.1	—	—	+	+
		0.5	—			—
		1.0	—			—
		3.0	—			—
	Tergitol-7	0.3	—			+
		0.5	—			+
		1.0	—			—

* A minus sign in the table indicates bactericidal action (no growth in subculture tube)

ment 5) Even at the very lowest concentrations of Metaphen and Merthiolate, however, lecithin did not protect.

Some experiments have been performed with the selective bactericidal agents isolated by Dubos (27) and by Hoogerheide (28).² Both preparations were dissolved in a small volume of alcohol and diluted with water to give a

² Gramicidin was purified from a preparation kindly supplied by Eli Lilly and Company. This probably contained several of the bactericidal substances described by Dubos and coworkers (29-31). We wish to thank Dr. J. C. Hoogerheide for a sample of his material.

TABLE VI

Effect of Soy Bean Lecithin on Inhibition of Bacterial Metabolism by Mercury Compounds

Experiment	Bacteria	Concentration of mercury compound			Inhibition by mercury compound alone	Inhibition by mercury compound + 3.0 mg lecithin
		HgCl ₂	Metaphen	Merthiolate		
		mg / 3 cc	mg / 3 cc	mg / 3 cc	per cent	per cent
1	<i>Staphylococcus aureus</i> *	0.05	0.05	0.05	89	88
					84	86
					76	74
2	<i>Sarcina lutea</i> *	0.10	0.10		92	60†
					91	58†
3	<i>Lactobacillus</i> ‡	0.05		0.10	89	90
		0.10			91	98
					91	98
4	<i>Micrococcus tetragenus</i> §	0.10			90	91
5	<i>Escherichia coli</i> *	0.05			91	85
		0.025			91	77
		0.010			90	11
		0.005			81	1
		0.001			6	5
			0.05		93	96
			0.025		93	94
			0.010		90	90
			0.005		78	61
			0.001		16	11
				0.05	82	83
				0.025	75	72
				0.010	63	70
				0.005	50	55
				0.001	13	8

* Respiration

† 6.0 mg lecithin

§ Aerobic glycolysis

stable, milky emulsion. Control experiments showed that the low concentration of alcohol present did not influence the results. It was found that lecithin could effectively prevent the inhibition of bacterial metabolism caused by these compounds (Table VII). Other phospholipids were also active. All the experiments reported in Table VII were performed with a phospholipid

concentration of 1 mg per cc No effort was made to determine the minimum effective phospholipid concentration

TABLE VII
Effect of Phospholipids on Inhibition by Gramicidin and Hoogerheide's Compound

Bacteria	Phospholipid	Concentration of inhibitor		Inhibition* by inhibitor alone	Inhibition* by inhibitor plus 3 mg phospholipid
		Gramicidin	Hoogerheide		
		mg /3 cc	mg /3 cc	per cent	per cent
<i>Staphylococcus aureus</i> †	Lecithin (beef)	0 2	—	—68	+4
	Lecithin (soy)	0 1	—	—45	+18
		0 2	—	—81	+53
		—	0 1	—51	+37
		—	0 2	—69	+29
		—	0 3	—73	+42
<i>Sarcina lutea</i> ‡	Lecithin (soy)	0 1	—	—83	+92
		—	0 2	—77	+40
Lactobacillus§	Lecithin (soy)	0 1	—	—99	—2
		—	0 1	—93	—9
	Lecithin (beef)	0 1	—	—91	—28
		—	0 1	—47	—17
	Cephalin (soy)	0 1	—	—93	—7
		—	0 1	—70	—9
		—	0 2	—97	+11
	Sphingomyelin	0 1	—	—90	—12

* Minus signs represent inhibition, plus signs represent stimulation

† Respiration

§ Aerobic glycolysis

Protective Action by Surface-Active Compounds Other than Phospholipids

An unionized detergent, Demal,³ was found to be very effective in preventing the inhibitory action of both cationic and anionic detergents. This detergent is described (32, 33) as a mixture of polyglycerol esters, with the following type formula $R-COO-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2OH$, in which R represents a long-chain alkyl radical. This compound possesses the typical polar-nonpolar structure of surface-active compounds. On the other hand, it differs markedly from the cationic and anionic detergents be-

³ A sample of this compound was kindly supplied by the Emulsol Company

cause it does not ionize Furthermore, it appears to have no effect on bacterial metabolism

TABLE VIII
Effect of Demal on Inhibition of Bacterial Metabolism by Detergents

Bacteria	Detergent	Concentration of detergent	Concentration of Demal	Inhibition
		mg / 3 cc	mg / 3 cc	per cent
<i>Staphylococcus aureus</i> †	Zephiran	0	3 0	-4
		0 05	0	-85
		0 05	3 0	0
		0 05	1 0	+12
		0 05	0 5	+11
		0 05	0 3	+3
	Tergitol 7	0 3	0	-85
		0 3	3 0	-14
<i>Escherichia coli</i> ‡	Zephiran	0	1 0	0
		0 05	0	-82
		0 05	1 0	-15
		0 05	0 5	-42
		0 05	0 3	-57
		0 05	0 1	-72
<i>Lactobacillus</i> §	Zephiran	0	1 0	0
		0 05	0	-77
		0 05	1 0	-9
		0 05	0 5	-11
		0 05	0 3	-32
		0 05	0 1	-83
	Tergitol 7	0	1 0	0
		0 05	0	-92
		0 05	1 0	-4
		0 05	0 5	-2
		0 05	0 3	-20
		0 05	0 1	-80
		0 05	0 1	-80

* Minus signs represent inhibition plus signs represent stimulation

† Respiration

§ Aerobic glycolysis.

The results obtained with this compound are illustrated in Table VIII. It can be seen that relatively low concentrations of Demal prevent inhibition of bacterial metabolism by both Zephiran (cationic) and Tergitol 7 (anionic).

It is of interest to note that other experiments (not included in Table VIII) demonstrate that the unionized detergent, Demal, also prevents the inhibition caused by gramicidin. Thus, in an experiment with *Lactobacillus*, 0.1 mg of

gramicidin inhibited aerobic acid production 99 per cent, in the presence of 3 mg of Demal, the inhibition was only 19 per cent

Some experiments were performed with cholesterol to determine whether it protected against the detergents in a manner similar to lecithin. Due to its insolubility in water, it was necessary to use alcoholic solutions. A concentration of 1.2 mg was unable to prevent the inhibitory action of 0.05 mg of Zephiran on *Staphylococcus aureus* or *E. coli*.

It is known that detergents of opposite ionic charge precipitate each other, and therefore the inhibition of bacterial metabolism by a cationic detergent can be prevented by the simultaneous addition of an anionic detergent, and *vice versa*. Thus, the action of a cationic detergent such as Zephiran can be prevented by the simultaneous addition of an equivalent quantity of an anionic detergent, decyl sulfate. On the other hand, a combination of anionic detergents, one of which is inhibitory and the other which is not, (such as Tergitol-7 and decyl sulfate), still inhibits bacterial metabolism. Similarly, the surface-active compound, sodium taurocholate, prevents the action of Zephiran but not of Tergitol-7, it behaves like a typical anionic synthetic detergent. The neutralization of oppositely charged detergents appears to be a different phenomenon from the protective action of the phospholipids and Demal.

Effect of Compounds Which Are Not Surface-Active

It was thought of interest to determine if compounds which are not surface-active but are known to be involved in growth or metabolism of bacteria or in some manner related to the phospholipids, could act like phospholipids in protecting bacteria against the action of detergents. In experiments on *Staphylococcus aureus* and lactobacillus (respiration and acid production studies, respectively) it was found that nicotinic acid and nicotinamide, thiamine, riboflavin, diphosphopyridine nucleotide,⁴ and yeast extract were unable to prevent the inhibition by Zephiran. Negative results were also obtained with the following compounds: methylene blue, choline, glycerol, and the ethanolamines (mono-, di-, and tri-).

DISCUSSION

It appears to us that the most reasonable working hypothesis to explain the rapid action of synthetic detergents on bacterial metabolism and viability would be one based on a twofold action: first, a disorganization of the cell membrane by virtue of the great surface activity of these compounds, and second, a denaturation of certain proteins essential to metabolism and growth. The effects of detergent-like compounds on lysis and agglutination of red cells have been investigated by Schulman (34). He concluded from studies on model systems that compounds which penetrate lipoprotein monolayers

⁴ We are indebted to Dr. A. Altschul for a sample of purified material.

increase surface pressure markedly and cause lysis, whereas compounds which do not penetrate but are adsorbed cause agglutination. It is conceivable that similar disorientations and alterations in surface forces may occur in bacterial cells. Denaturation of proteins and inactivation of viruses have been reported by a number of investigators (3, 4, 35-40). Very low concentrations of detergents have been shown to denature proteins.

If such an explanation were correct, then it is reasonable to expect that compounds which could significantly alter the affinity of detergents for bacteria would influence their effect on the cell membrane and their tendency to denature cell proteins. Phospholipids possess a characteristic polar nonpolar structure and, presumably, have an affinity for bacterial cells similar to that of the detergents. They have been shown to produce a marked lowering of surface tension at very low concentrations (41). Since they do not inhibit bacterial metabolism even at quite high concentrations, the phospholipids could protect the bacterial cell, perhaps by altering the structure of the membrane in such a manner as to prevent penetration by the detergents. As some evidence for this it should be noted that our experiments demonstrate that the phospholipids are ineffective unless they are added before or at the same time as the detergent. It will be of interest to establish whether or not the phospholipids can prevent denaturation of proteins by detergents.

We have found that an unionized detergent, Demal, functions very similarly to the phospholipids protecting bacteria against both cationic and anionic detergents. On the other hand, the action of taurocholate is confined to cationic detergents, and cholesterol is relatively inactive.

We have not had the opportunity to study phospholipids derived from bacterial cells. Such an investigation would be of value in elucidating a possible relationship between cellular phospholipids and the resistance or susceptibility of various cells and bacterial species to the detergents.

There is a striking contrast between the action of phospholipids against the synthetic detergents and the bactericidal compounds of Dubos and Hoogerheide, as compared with the very low activity of phospholipids against mercuric salts and derivatives. Fildes (42) has demonstrated that mercuric ions act on bacterial cells by combining specifically with sulfhydryl groups. The inhibitory action can be reversed even after long periods of time by the addition of sulfhydryl compounds which form soluble compounds with mercury, such as glutathione, cysteine, and thiolacetate. As shown above, the action of the detergents on bacteria appears to be of a different type, and does not seem to involve combination with a specific group.

SUMMARY

1. Lecithin, cephalin, and sphingomyelin prevent the inhibition of bacterial metabolism which is caused by synthetic anionic and cationic detergents. The phospholipids must be added either before or simultaneously with the deter-

gent Addition after the detergent is without effect Bacteria still exhibit this phenomenon after they have been exposed to the phospholipid and thoroughly washed

2 A similar action of the phospholipids has been demonstrated towards the bactericidal compounds isolated by Dubos and Hoogerheide from soil bacteria There is very little effect with bactericidal mercury compounds

3 The effect of lecithin against the *bactericidal action* of synthetic detergents was also determined It was found that germicidal quantities of the detergents were not effective in the presence of the phospholipids

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sibility If there were certain tissues from which sodium and thiocyanate disappear at a slower rate than from other portions including the serum, then, following the second injection, less radiosodium and thiocyanate will diffuse into these particular tissues than after the first injection, and the concentration in the serum will be higher than it should be As will be pointed out later, the skeleton and central nervous system may serve as such tissues for sodium

Effect of Seasonal Variation upon the Volume of the Blood and Extracellular Fluid—Measurements of the plasma volume and the extracellular fluid were made on five normal subjects in Rochester during the warm season and again during the winter months In the month of March, 1940, during which time all but one of our observations were made, the mean daily high temperature

TABLE IV

The Effect of Seasonal Variations upon the Volume of the Blood and the Sodium Space

Normal subjects	Date	Time after injection	Serum protein	Cells	Plasma volume		Blood volume		Interstitial fluid		Sodium space	
					Liters	Difference	Liters	Difference	Liters	Difference	Liters	Difference
		hrs	gm per cent	per cent		liters		liters		liters		liters
L Y	Mar 1940	3	6.7	45.0	3.31		6.02		16.8		20.1	
	Aug 1939	3	6.6	43.8	3.51	+0.20	6.25	+0.23	17.6	+0.80	21.1	+1.00
F B	Mar, 1940	3	6.4	49.6	2.84		5.64		18.5		21.3	
	Sept 1939	3	6.2	48.0	2.97	+0.13	5.72	+0.08	18.7	+0.20	21.7	+0.40
J D	Mar 1940	3	6.7	47.5	3.07		5.85		13.6		16.7	
	Sept 1939	3	6.9	46.8	3.06	-0.01	5.75	-0.10	14.2	+0.60	17.3	+0.60
V D	Apr 1940	3	6.2	44.0	2.99		5.34		14.6		17.6	
	Sept 1939	3	5.7	42.6	3.22	+0.23	5.61	+0.27	11.7	-2.90	14.9	-2.70
J A	Mar 1940	3	6.3	45.2	2.73		4.98		14.9		17.6	
	Sept 1939	3	6.3	44.8	2.50	-0.23	4.53	-0.45	13.3	-1.60	15.8	-1.80

was 33°F and the mean of the lows 22°F The mean relative humidity at 7 30 a m was 84 per cent, at 1 30 p m, 69 per cent, and at 7 30 p m, 78 per cent The percentage of sunshine was 47 Between August 9, and September 9, 1939, the mean daily high temperature was 80°F and the mean of the lows, 63°F, with an average relative humidity of 86 per cent at 7 30 a m, 51 per cent at 1 30 p m, and 66 per cent at 7 30 p m The percentage of sunshine was 74

During this study the subjects followed their ordinary activities and were exposed to the outside temperature only several hours a day The results are given in Table IV During the warm season, all the subjects weighed less than during the winter months (average difference 1.9 kg) During the warm weather the plasma volume increased in three subjects, remained the same in one, and decreased in two The mean difference in the plasma volume was +0.064 liter or +2.2 per cent with a range of from -11 per cent to +8 per

cent The mean difference was not significantly different from zero There was no significant change in the blood volume, the mean difference being $+6$ ml The changes in the sodium space were no more consistent than those found for the volume of the blood The volume during the warm season as compared with the cold increased in three subjects and decreased in two The mean difference was -0.50 liter, a value not significantly different from zero

These results are not in agreement with the marked changes in the volume of the blood found by Barcroft *et al* (15) and Bazett and coworkers (16), during seasonal variation The former investigators using the CO method for determining the volume of the blood found an increase of about 35 per cent in this volume as they sailed through the tropics and Bazett *et al* observed equally large increases in the blood volume during the summer heat There was an increase in both the plasma and the cell volume in the summer, so that the hematocrit readings did not change More recently Forhes Dill, and Hall (17) found much less marked changes in the blood volume with changes in climate A group of laboratory workers on moving to a hot damp climate for the summer showed an increase in the volume of the blood and plasma of only 4.5 and 4.2 per cent, with a range from -6 per cent to $+12$ per cent

We have employed essentially the same methods for the blood volume as Forhes and his group The results in the two groups are very similar The discrepancy between the results of Barcroft and Bazett and Forhes and ours cannot be explained at the present time

DISCUSSION

The results of this investigation indicate that the fluid available for the distribution of radiosodium (sodium space) represents approximately 25 per cent of the body weight The sodium space will be equal to the volume of extracellular fluid providing that either sodium is exclusively limited to the extracellular phase, or if not, it does not exchange with intracellular sodium, and further providing that the radioactive isotope is equally distributed throughout the sodium phase There is good evidence to suggest that neither sodium nor chloride is exclusively limited to the extracellular compartment Manery and Hastings (13) have shown that the Na:Cl ratio varies in different organs The ratio for most tissues is approximately the same as that of an ultrafiltrate of serum, but in certain tissues (stomach, tendon, and testes) there is excess chloride while in certain other tissues (spinal cord, cartilage, and bone) there is extra sodium which suggests the existence of both intracellular sodium and chloride The results of Harrison Darrow, and Yarnet (18) on the analysis of whole bodies of animals support these findings with respect to sodium They found an excess of sodium amounting to ap-

proximately 25 per cent of the total sodium in the dog, 85 per cent of which was limited to the skeleton

Several questions then arise in reference to the use of marked sodium as a means of measuring extracellular fluid. First is the tagged sodium distributed in the same proportion as total sodium, and second does the radiosodium diffuse into the skeleton and reach its maximum concentration during the 12 hours allotted for mixing in man? In answer to the first question, Greenburg *et al* (19) and Manery and Bale (20) observed that radiosodium penetrates all the tissues and is distributed in the same proportion as total sodium. Radiosodium is not concentrated in any tissue. A definite answer to the second query cannot be given. Manery and Bale (20) found that in certain tissues of rabbits and rats the penetration of radiosodium was complete 20 minutes after the injection and remained constant for at least 12 hours, and that in certain other tissues (testes, brain, and bone) the penetration was delayed but gradually proceeded to completion in 3 to 12 hours. Their data on bone were very sparse. Greenburg *et al* (19) using rats found that the marked sodium diffused rapidly throughout the tissues. Their studies did not include bone. Hahn, Hevesy, and Rebbe (21) observed that 67 hours after the subcutaneous injection of radiosodium in a rabbit the concentrations of marked sodium in tibia epiphyses and tibia diaphyses were 59.1 and 50.9 per cent of that in plasma respectively.

These observations then suggest that in the series of experiments reported above the total sodium of the body was measured, and therefore the figures given for the volume of extracellular fluid are too high, because of excess sodium attributed to the extracellular volume.

In order to determine how rapidly sodium diffuses into bone and what concentration it reaches, the experiments of Harrison *et al* (18) have been repeated in part on dog and man using radioactive sodium rather than chemical methods.

After securing a sample of serum to serve as control, radiosodium and sodium thiocyanate were injected intravenously. At suitable intervals thereafter samples of blood and bone were obtained, the β -ray activity counted, and the chloride concentration chemically determined. The bone was freed from excess tissue (fat, marrow) and weighed. One portion was used for the determination of chloride, and another was dissolved in concentrated nitric acid on a steam bath. 2 ml of the resultant solution were placed in a Geiger-Müller counter and its activity measured. The first two dogs were sacrificed 12 hours after the injection of the substances and numerous samples of bone were obtained. In the third dog, after the substances were injected, the animal was kept in an anesthetized state with nembutal during the remainder of the experiment. Every 3 hours for 12 hours after the administration of sodium, samples of blood were obtained and at each period corresponding sections of tibia or radius were removed using a different limb at each period.

The counts of the tissues alone mean very little, but their relative values give information desired, when related to the number of counts in the serum. The ratios of the tissue concentration to the plasma concentration for radioactive sodium and the chemically determined chloride were calculated. When the ratios are corrected for the water content of serum and the Gibbs Donnan equilibrium the resulting values give the apparent volume of the extracellular water. In keeping with the symbols used by Manery and Bale (20) the calculated value has been designated $(H_2O)_E$.

The results of these experiments along with data secured from humans are presented in Table V. The values for $(H_2O)_E Na^{24}$ in bone in every instance were much higher than those for $(H_2O)_E Cl$, indicating that there is extra sodium in bone which cannot be attributed to the extracellular compartment. The average values for $(H_2O)_E Na^{24}$ in bone correspond very closely to the value of 52 calculated from the results of Harrison *et al* (18). In general the figures for $(H_2O)_E Na^{24}$ were higher in compact than in cancellous bone. Another finding of interest is that the value for $(H_2O)_E Na^{24}$ in bone of dog 3, 3 hours after the injection was just as high as that found at the end of 12 hours. At least in the dog, radiosodium penetrates bone and diffuses into it rather rapidly.

The nature of the "excess sodium" in bone is not known. It is felt that the extra sodium must contribute little, if any, to the osmotic pressure of skeletal water and presumably is present as an insoluble or undissociated compound serving to form the matrix of bone. Harrison *et al* (18) felt that the excess sodium of bone was associated with the inorganic part. We attempted to find out the percentage of radiosodium associated with the organic and inorganic portions of bone. Counts were made on adjacent portions of bone, after treating one part with nitric acid and the other after extracting the organic part of the bone with ethylene glycol. The results show that the counts associated with the organic part of dense bone 12 hours after injection represented 56.5 per cent of the total count, while those of spongy bone represented on an average 73 per cent of the total activity. However, these data do not throw much light on the nature of the extra sodium in bone because of the relatively short duration of these experiments. The rate of penetration of the radiosodium into the inorganic compound may be governed by the rate at which sodium is renewed.

From the information at hand it is possible to calculate the excess radio sodium that diffused into bone of the dog. Using 24.3 per cent of the body weight as skeleton (18) and 60 for $(H_2O)_E Na^{24}$ in bone in dog 1 it was found that 41.6 per cent of the number of counts present in the body were in bone. This compares favorably with Harrison's value of 46 per cent determined chemically. Assuming that the chloride space of bone (20 per cent of its weight) represents its extracellular volume, then the extra sodium in bone

TABLE V

Ratios of Tissue (Bone) Concentration to Plasma Concentration for Radioactive Sodium and Chemically Determined Chloride

	Compact bone						Cancellous bone	Compact and cancellous bone	Spinal cord				
	Femur		Scapula	Radius tibia		Humerus		Femur epiphysis scapula		Vertebra			
	(H ₂ O) _T Na ²²	(H ₂ O) _T Cl	(H ₂ O) _T Na ²²	(H ₂ O) _T Na ²²	(H ₂ O) _T Cl	(H ₂ O) _T Na ²²	(H ₂ O) _T Cl	(H ₂ O) _T Na ²²	(H ₂ O) _T Cl	(H ₂ O) _T Na ²²	(H ₂ O) _T Cl		
Dog 1	66.4 59.5 66.8	23.5 22.1	72.0 75.3					45.2 32.9 53.9	32.9 26.3				
Dog 2	56.9 58.4 51.2 62.2	20.1 21.8						31.4 36.7	17.1 17.7	39.9		29.3	44.6
Dog 3	72.6	20.0 21.0		68* 64.2 68.7† 64.3 71.4‡ 75.4 66.4 64.5	20.0	62.9 62.9	18.2	46.3	20.4	51.0	24.4	—	46.0
Patient H H										Skull 48.9 34.6			
M A		19.6 22.8											
A S			Rib 51.4 41.5 44.0										
W S										Spine 56.5 62.5			

* Portion of tibia, diaphysis removed at 3 hours

† Portion of tibia, diaphysis removed at 6 hours

‡ Portion of radius, diaphysis removed at 9 hours All other samples obtained 12 hours after injection of radiosodium

$$(H O)_E Na^{22} = \frac{\text{Tissue counts per min per kilo}}{\text{Serum counts per min per liter}} \times \frac{0.93}{0.95} \times 100$$

$$(H O)_E Cl = \frac{\text{Tissue Cl (m eq per kilo)}}{\text{Serum Cl (m eq per liter)}} \times 0.93 \times 0.95 \times 100$$

was equal to 32 per cent of the total counts in the body The average value for three dogs was 28.5 per cent If Skelton's value (22) of 17.4 per cent of the body weight as skeleton is employed, then 29.8 per cent of the total number of

counts was found in the bone (dog 1) and the excess counts amounted to 20 per cent of the total counts remaining in the body. The average value (three dogs) for excess counts was 18.1 per cent. The extent then to which the excess sodium in bone will correct the sodium space, depends on the value selected to represent the percentage of skeleton to body weight. The value given by Skelton (22) probably represents a closer approximation to the conditions of these experiments than Harrison's values, since the bone used for analysis was free of excess tissue and fat.

Employing Skelton's figure, the sodium space of the dogs was corrected for the excess sodium existing in bone (Table VI). The corrected volumes were similar to those determined by the distribution of thiocyanate. The average corrected sodium space of the three dogs was 4.8 liters or 27.6 per cent of the

TABLE VI

Fluid Available for the Distribution of Radiosodium Corrected for the Amount of Extra Sodium in Bone (Dogs)

Dog No	Time after injection	Weight	$(\text{H}_2\text{O})_E \text{Na}^{24}$ (Bone)	Sodium space	Sodium space corrected		Thiocyanate space	
					Liters	Body weight	A†	B†
	hr	kg		liters		per cent	liters	liters
1	12	15	60.0	5.26	4.22	28.1	5.23	5.01
2	12	19	47.6	6.14	5.23	27.5	5.81	5.32
3	3	17.9	58.4	6.35	5.11	28.6	5.25	4.80
	6			6.22	5.00	27.9	5.30	5.08
	9			6.38	5.12	28.6	5.59	5.19
	12			6.06	4.86	27.2	5.57	5.17

* Corrected for excess sodium in skeleton

† See Methods

body weight. These values are similar to those derived by more direct methods (18). No correction has been applied for the diffusion of labeled sodium into the erythrocytes (23).

Similar results were found in man. The values for $(\text{H}_2\text{O})_E \text{Na}^{24}$ were higher than those for $(\text{H}_2\text{O})_E \text{Cl}$. In two instances (A S and W S) in which ample samples of bone were obtained, the values for $(\text{H}_2\text{O})_E \text{Na}^{24}$ were similar to those found for the dog (Table V). In one case (A S) in which the data were complete, it was found that 37.4 per cent of the total number of counts in the body was in bone and that the excess sodium in bone was equivalent to 21 per cent of the total sodium counts.

Corrections for the sodium space were made in ten normal subjects (Table VII). It was assumed that the skeleton represented 16 per cent of the body weight (22) and the values of 50 and 20 were taken for $(\text{H}_2\text{O})_E \text{Na}^{24}$ and $(\text{H}_2\text{O})_E \text{Cl}$ respectively. In eight normal subjects in whom both sodium and thiocyanate spaces were determined, the correction applied reduced the volume

determined by sodium an average of 3.7 liters or 18.9 per cent. The average corrected value for the sodium space was 15.9 liters (21.1 per cent of the body weight) compared with 17.7 liters (23.5 per cent of the body weight) for the corrected thiocyanate space. These values then represent the closest approximation to the volume of extracellular fluid that one can make with the available data. Both the sodium and thiocyanate spaces require large corrections, the former for excess sodium in bone and the latter for thiocyanate in erythrocytes.

TABLE VII

Fluid Available for the Distribution of Radiosodium Corrected for the Amount of Extra Sodium in Skeleton (Normal Subjects)

Experiment No.	Subject	Time after injection	Weight	Interstitial fluid (radiosodium)		Extracellular fluid sodium space		Extracellular fluid thiocyanate space	
				Volume	Corrected* volume	Volume	Corrected† volume	A*	B*
		hrs	kg	liters	liters	liters	liters	liters	liters
5	J. T.	12	51.0	14.8	12.3	17.5	15.0		
6	J. P.	12	69.8	15.8	12.5	18.4	15.0		
7	L. Y.	6	84.5	17.6	13.6	20.9	16.9	20.6	19.4
8	F. B.	6	102.5	19.4	14.5	22.2	17.3	21.0	19.8
9	V. D.	6	61.3	15.2	12.2	18.2	15.2	17.1	16.0
10	J. A.	6	67.3	15.6	12.4	18.3	15.1	18.2	17.2
11	G. M.	6	106.3	21.2	16.1	25.5	20.4	24.4	22.9
12	S. G.	6	56.8	12.9	10.2	15.3	12.6	15.1	14.2
		8		12.8	10.1	15.2	12.5		
13	N. K.	6	77.6	16.6	12.9	20.5	16.8	19.7	18.2
		9		17.2	13.5	21.1	17.4		
		12		17.3	13.5	21.2	17.4		
14	J. C.	6	58.8	13.2	10.4	15.6	12.8	15.0	14.1
		9		14.2	11.4	16.6	13.8		
		12		14.1	11.3	16.5	13.7		
Average		6		16.5	12.8	19.6	15.9	18.9	17.7
Body weight per cent				21.8	17.0	25.9	21.1	25.0	23.5

* See "Methods"

† Corrected for excess sodium in skeleton

Since the major portion of the extra sodium of the body is contained in skeleton (18, 20), the correction applied to the sodium space will depend on the weight of the skeleton. Due to the variability of the relative weight of the skeleton to the body weight in different individuals and in the same individuals under different conditions, it is impossible to arrive at the absolute weight of the skeleton and in turn to apply to the sodium space proper corrections for extra sodium.

Radioactive sodium may be employed in making comparative measurements of the sodium space on the same individual at two different times, providing the store of extra sodium does not fluctuate with the expansion or the contrac-

tion of the extracellular volume or with changes in the concentration of sodium in the extracellular fluid. Due to the nature of bone, one should expect little change in its sodium content with fluctuations in the volume of extracellular fluid without changes in the sodium concentration. It is conceivable, however, that drastic changes in the concentration of sodium in the extracellular fluid may vary the amount of extra sodium in the body. In the salt deficiency experiments done by McCance it appears that the subject R A M lost approximately 210 m eq of sodium which came from some other portion of the body than his extracellular fluid (24).

It appears then that neither radiosodium nor thiocyanate is an absolute measure of the extracellular fluid. Since sodium and chloride are not exclusively limited to the extracellular phase, the volume of fluid available for radiosodium and thiocyanate is larger by an undeterminable amount than the space which functions as the interstitial fluid. In general, in soft tissues, chloride exceeds sodium while in skeleton sodium exceeds chloride (13). Radioactive sodium affords then at least as accurate a method as any previously described. Since our main interest in measuring the extracellular compartment is for comparative purposes in the same individual at two different times and not necessarily the absolute volume, the most useful representation of this volume at the present time is as follows:

$$\text{Sodium space, liters} = \frac{\text{Counts injected} - \text{counts lost in urine}}{\text{Concentration of counts per minute per liter of serum}}$$

In the derivation of this equation correction for the Gibbs Donnan effect and the water content of serum was considered, but the values for these factors, 0.95 and 1/0.94 respectively, for practical purposes cancel each other. Cancellation of these factors results in a sodium space which is 200 cc too high, providing we assume that the interstitial fluid of the normal subject is free of proteins. The futility of attempting to correct the concentration of counts of serum more closely than this is obvious when one recalls that the protein content of interstitial fluid of normal man is not known and that in patients with edema and serous effusions the transudates contain variable amounts of proteins not only in different subjects but also in different parts of the extracellular compartment in the same patient.

Finally it is worth while to point out the relative merits of radiosodium and thiocyanate as measures of the volume of extracellular fluid. Thus far there has been no evidence presented to show that radiosodium differs either chemically or physiologically from the sodium which occurs in nature (25). The evidence indicates that thiocyanate and tagged sodium spread from the plasma into their respective phases of individual tissues in the same proportion as chloride and total sodium are distributed. In normal man diffusion equilibrium of thiocyanate is more rapidly attained than that for sodium. From

the simultaneous measurements made on serum and serous effusions, it appears that from 9 to 12 hours is necessary for complete diffusion of sodium and thiocyanate in the bodies of patients suffering from congestive heart failure (7) Radiosodium has the advantage over thiocyanate in that it measures the volume of extracellular fluid of the nervous system, and it is not concentrated in the digestive juices Radiosodium does not enter the red blood cells to any great extent as does thiocyanate (26) Neither radiosodium nor thiocyanate is rapidly excreted, so sufficient time (12 hours) may be allowed between the administration of the substances and the drawing of blood samples in order to insure complete diffusion through large accumulations of fluid The amount of sodium chloride that it is necessary to inject in order to make accurate measurements is not large enough to cause changes in the electrolyte balance One disadvantage is that labeled sodium is not always available and that it requires considerable technical equipment which is expensive

SUMMARY AND CONCLUSIONS

A method for measuring the volume of fluid available for the distribution of sodium (sodium space) by the use of its radioactive isotope (Na^{24}) has been described and the accuracy of the method has been discussed Simultaneous determinations of the plasma volume by means of the blue dye T-1824 and the volume of the extracellular fluid by employing radiosodium and sodium thiocyanate have been made in normal subjects Repeated measurements were made at varying periods of time in the same individuals In order to establish the rate of diffusion equilibrium for the radioactive isotope of sodium and thiocyanate between serum and serous effusions, simultaneous samples of both were obtained at varying intervals after the intravenous injection of these substances

Since evidence in the literature indicates that there is an excess of sodium mainly limited to bone, which cannot be attributed to the extracellular phase, experiments on dogs and man were so devised that the ratio of tissue concentration to plasma concentration for radiosodium and chemically determined chloride could be calculated

The following conclusions may be drawn from the results of this investigation

- 1 Radiosodium after intravenous administration spreads rapidly during the first 3 hours from the plasma into a volume of fluid which represents approximately 25 per cent of the body weight of man Thereafter for 6 hours it diffuses more slowly into certain tissue spaces—the central nervous system and probably the skeleton The plasma volume and interstitial fluid represent 15 and 85 per cent of the sodium space respectively

- 2 Diffusion equilibrium for both radiosodium and thiocyanate is not established between serum and transudates in edematous patients until from 9 to 12 hours after the intravenous injection of these substances

3 Until more complete information is available, it is concluded that unless the difference between repeated observations on the same individual exceeds ± 1.38 liters there is no significant change in the sodium space providing that the activity of the standard and serum samples are in the range of 40 counts per minute per milliliter with the counting apparatus used. As the activity of the samples increases, the error becomes less because there is no correlation between the magnitude of the error and the magnitude of the activity.

4 Climatic conditions produce no significant changes in the volume of the blood or extracellular fluid.

5 In the dog, following the intravenous injection of radiosodium, the concentration of the isotope in bone reaches its maximum rapidly (3 hours). The extra sodium in the skeleton of dog is equal to about $\frac{1}{2}$ of the total counts in the body, assuming that the chloride space of bone represents its extracellular volume. Similar amounts of excess sodium are found in the skeleton of man 12 hours after the administration of Na^{24} .

6 Correction of the sodium space of man for the excess sodium reduced the average value by 3.7 liters or 18.9 per cent. The average corrected volume for the normal subjects 6 hours after the injection is 15.9 liters or 21.1 per cent of the body weight compared with the thiocyanate space of 17.7 liters, representing 23.5 per cent of the body weight.

7 The most useful method for calculating the sodium space from the data obtained after intravenous administration of radiosodium is as follows:

$$\text{Sodium space, liters} = \frac{\text{Number of counts retained in the body}}{\text{Concentration of counts per minute per liter of serum}}$$

This space exceeds the volume of extracellular fluid by the amount of excess sodium in the body that cannot be attributed to the extracellular phase.

8 While neither the thiocyanate method nor the radiosodium method gives precise estimates of the extracellular fluid, the error is of the same order of magnitude in both. For clinical use, the thiocyanate method is superior because of the ready availability of the substance, and the apparatus required.

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THE EFFECT OF HIGH PROTEIN DIETS ON EXPERIMENTAL RENAL HYPERTENSION*

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Although the belief is widely held that a high protein diet is deleterious to the hypertensive patient, a careful survey of the literature discloses that this is not a generally substantiated point of view. Some clinicians (1-4) in particular Newburgh (5) have insisted that the withdrawal of protein from the diet of patients with hypertension results in the reduction of their blood pressure and the elimination of the concomitant symptoms. Others have maintained that the amount of protein in the diet generally has little or no relation to the blood pressure (6, 7). It has even been suggested that a continued low protein diet may be pathogenic (8, 9).

The relationships between renal excretory insufficiency and diet have been studied experimentally by numerous investigators (10), but the recent development of a satisfactory method for the experimental production of persistent renal hypertension (11) has made possible studies on the relationship between the factors of diet, renal excretory insufficiency and hypertension of renal origin in animals (12, 13).

Although the processes which result in relative renal excretory insufficiency and those factors which lead to hypertension are frequently seen together, we have shown that they are fundamentally distinct (14, 15). Nevertheless there still remains the possibility that an augmented load on the kidney might cause an increased amount of work and thus lead to a relative renal ischemia and the induction of those processes which lead to hypertension. We have therefore undertaken a study of the effect of high and low protein diets upon dogs with (a) normotension and unimpaired renal function, (b) normotension and embarrassed renal function due to temporary complete occlusion of the renal arteries, (c) normotension and relative renal excretory insufficiency due to chronic partial occlusion of the renal arteries, (d) moderate hypertension with slight evidence of renal excretory insufficiency, (e) severe hypertension with frank renal excretory insufficiency.

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TABLE I
Summary of Pertinent Data on Effects of Diet Changes

Dog	Diet	Days on diet	Blood N.P.N. mg %	Blood pressure			
				Ave mm Hg	Max mm Hg	Min mm Hg	Last mm Hg
V-43 * normal pregnant no renal impairment normotensive	Standard	5		145/75	150/80	140/70	140/70
	Bread and milk	24		160/85	175/95	120/60	155/90
	Meat	30		165/90	180/100	160/75	150/100
	Standard	37	27	150/75	150/80	140/70	140/70
	Meat and salt	10		130/80	135/85	120/75	125/85
	Standard	3		125/80			120/75
Y 1 † uninephrectomy contralateral temporary renal artery occlusion for 2 hrs moderate renal failure normo- tensive	Standard	53		170/100	190/115	150/85	190/110
	Bread and milk	30	47	180/100	200/120	170/95	160/105
	Meat	27	98	170/100	175/100	165/85	165/85
			88				
	Bread and milk	16	56	195/105	210/110	175/90	175/90
			38				
Y-80, § temporary renal artery occlusion for 2 hrs moderate renal failure normotensive	Standard	25	54	155/85	170/100	150/70	160/95
	Bread and milk	25	96	150/75	165/80	130/70	150/75
			58				
	Meat	30	124	165/80	155/100	150/75	175/75
			96				
			59				
W-67 uninephrectomy with contralateral par- tial renal artery occlu- sion kidney function partially impaired nor- motensive	Bread and milk	18	41	160/80	175/95	150/70	150/70
			46				
	Standard	17	39	180/90	170/90	175/85	175/75
	Bread and milk	15	31	140/75	155/90	125/50	155/70
	Meat and salt	11	56	155/80	185/100	100/50	165/100
	Bread and milk	11	39	150/80	160/90	140/75	160/85
W-87 bilateral partial renal artery occlusion, kidney function par- tially impaired normo- tension	Meat	9	64	160/90	165/95	150/80	160/85
	Bread and milk	7	30	165/75	175/75	150/75	175/75
	Standard	13	31	155/85	175/95	150/70	150/75
	Bread and milk	18		145/75	160/100	130/70	160/90
	Meat and salt	10	47	170/80	170/85	120/75	120/75
	Bread and milk	12	58	160/85	170/100	125/75	125/85
U-45 uninephrectomy contralateral kidney ex- planted partially impair- ed renal function due to trauma to explanted kidney normotension	Meat	17	55	145/95	175/100	125/75	150/95
	Bread and milk	2	35	145/75	160/75	130/75	130/75
	Standard	20	45	170/80	165/85	190/75	150/80
	Bread and milk	7		165/90	160/100	150/70	170/85
	Meat and salt	12	70	165/90	175/100	155/75	165/85
	Bread and milk	9	36	165/90	175/85	145/90	170/90
W 77 bilateral partial renal artery occlusion moderate impairment of renal function normo- tensive	Meat	9	52	175/95	175/100	170/85	175/100
	Bread and milk	3	49	185/100	190/100	180/100	190/100
	Standard	12	38	140/75	160/85	125/75	125/70
	Bread and milk	17	45	140/70	150/75	125/65	150/75
	Meat and salt	11	66	150/80	165/85	125/75	125/75
	Bread and milk	12	38	145/80	145/85	140/75	150/75
	Meat	15	92	170/100	170/100	175/95	175/95
	Bread and milk	16		155/75	165/75	150/75	150/75
	Standard	33		140/80	120/85	130/70	130/70
	Bread and milk	61	37	145/80	170/100	140/65	175/85
			41				
	Meat	32	84	150/85	175/100	140/75	165/90
	Bread and milk	17	46	160/90	180/100	150/75	175/100
	Standard	24		150/80	175/85	125/70	160/85
	Bread and milk	25	45	160/85	175/110	150/70	150/70
	Meat	21	72	150/80	155/90	150/70	145/70
			63				
	Bread and milk	16	35	150/75	150/90	125/70	125/70
			36				
	Standard	1					175/95

TABLE I—*Concluded*

Dog	Diet	Days on diet	Blood N.P.N	Blood pressure			
				Ave.	Max	Min	Last
			mg	mm Hg	mm Hg	mm Hg	mm Hg
Y 9 bilateral partial renal arterial occlusion Kidney function unimpaired renal hypertensive	Standard	43	32	160/95	175/100	150/80	165/90
	Bread and milk	60	31	160/95	195/120	140/75	140/75
	Meat	33	38	165/100	140/125	140/80	120/105
	Bread and milk	12	30	175/110	175/120	175/100	175/120
	Standard	5		175/105	175/105	175/105	175/105
X 29 uninephrectomy with contralateral partial renal artery occlusion kidney function partially impaired renal hypertensive	Standard	21	44	180/100	230/130	140/85	140/85
	Bread and milk	5		170/110	250/150	155/100	165/105
	Meat and salt	11	54	160/100	185/125	145/80	175/110
	Bread and milk	9	55	130/95	165/105	140/80	155/90
	Meat	9	70	165/100	185/115	135/80	185/115
	Bread and milk	4	42	165/85	170/85	100/75	170/85
V 53 bilateral partial renal arterial occlusion Kidney function slightly impaired renal hypertensive	Standard	16		200/105	200/110	200/105	200/110
	Bread and milk	69	35	170/110	250/150	155/100	165/105
			41				
	Meat	32	34	175/110	195/125	160/95	175/100
	Bread and milk	18	46	180/110	185/115	170/100	170/100
	Standard	5		175/105	175/105	175/100	175/105
U 39 bilateral partial renal artery occlusion renal function unimpaired renal hypertension	Bread and milk	23	36	195/115	230/135	185/100	225/130
	Meat	32		190/115	210/125	190/105	185/110
	Standard	31		195/115	200/125	180/100	200/110
V 55 bilateral partial renal arterial occlusion Kidney function unimpaired renal hypertensive	Standard	21		195/110	205/125	195/95	195/95
	Bread and milk	61	27	190/120	220/130	170/100	170/105
			37				
	Meat	32		200/125	225/150	175/105	225/130
	Bread and milk	15	27	200/120	200/125	205/115	205/115
X-64 bilateral partial renal artery occlusion kidney function partially impaired renal hypertensive	Standard	18	37	200/120	200/120	205/115	190/120
	Bread and milk	43	38	190/110	200/125	175/75	180/100
	Meat	33	43	185/110	200/125	170/95	185/110
	Bread and milk	7		200/115	200/125	195/100	200/110
X-92 bilateral partial renal artery occlusion moderately severe renal insufficiency renal hypertension	Standard	8		225/140	250/150	235/130	250/150
	Bread and milk	64	40	210/125	230/150	200/100	200/100
			44				
	Meat	19	62	250+/160	280/185	200+/135	250/135
	Bread and milk	35	102	265/145	275/170	235/125	255/125
			40				
	Meat	9	86	190/155	245/155	180/150	180/150
	Bread and milk	8	82	245/155	230/175	230/125	250/145
			44				
	Standard	25	55	240/145	250/160	230/125	250/150
	Bread and milk	52	36	225/140	200+/165	200/125	200/125
	Meat	24		230+/150	225+/160	230+/130	230+/130
	Bread and milk	18	49	255/140	250/150	250/125	250/150
			41				
			38				
	Standard	2		215/135			225/150

* V-43 pregnant (near term) while on meat and salt

† Y 1, refuses meat when N.P.N. is 98 mg per cent and on meat regime N.P.N. falls to 56 mg per cent

§ Y 80 as in Y 1 refuses meat when N.P.N. is 124 mg per cent and N.P.N. then gradually falls to 96 and finally 59 mg per cent on meat regime

|| W-67 while on meat alone is in estrus

** V 92 on first regime of meat N.P.N. goes to 62 mg per cent and dog has uremic symptoms. The N.P.N. on the first day of bread and milk is 102 mg per cent. On the second meat regime the N.P.N. rose acutely to 86 mg per cent the dog was very sick with advanced uremic symptoms and diet was changed to bread and milk. On the third regime of meat the N.P.N. does not rise sharply the dog now refuses meat during periods of azotemia

In order to give the high and low protein diets an adequate period to influence the blood pressure and non-protein nitrogen of the blood the experimental animals were maintained on each diet for varying periods up to 2 months before the dietary regime was altered

Methods

Blood pressures were determined at least triweekly on trained unanesthetized dogs by direct arterial puncture with the Hamilton manometer (16). The method of training and the range of normal blood pressures have previously been described (17). Particular attention was given to the diastolic pressure since this is the more reliable index of the state of peripheral resistance. Blood non-protein nitrogen was determined according to the method used at this hospital (18).¹

Hypertension was induced by partial occlusion under nembutal anesthesia of the renal arteries with either Goldblatt clamps or linen ligatures (11, 19). Dogs were considered hypertensive when the diastolic blood pressure was maintained at a minimum of 40 to 50 mm Hg above the control level. The possibility that some of the more severely hypertensive animals were already maximally responding to the hypertensive stimulus was obviated by using several animals with a mild form of hypertension, *i e*, with a diastolic blood pressure of 20 to 30 mm Hg above the control level.

Renal excretory insufficiency without hypertension was induced by complete occlusion of the renal arteries for a period of about 2 hours. After release of the occlusion relative renal excretory insufficiency ensued (20). Chronic relative renal excretory insufficiency was considered to be present when the blood non-protein nitrogen was consistently above the normal level of 25 to 35 mg per cent, or could be significantly raised above that level by an increased protein intake.

The standard diet used for feeding our normal and hypertensive animals consists of scraps of vegetables, meat, bread, and milk returned from the hospital commissary. Representative samples indicate that approximately 35 to 40 gm of protein are in such a daily ration for each dog. The low protein diet consisted of powdered milk and white bread and contained about 15 gm of protein per dog per day. The high protein diet consisted of 800 gm of ground beef chuck containing 160 gm of protein per dog per day. In six experiments 10 gm of sodium chloride were added to the daily meat ration for each dog.

RESULTS

The essential data are summarized in Table I and illustrative experiments are shown in Figs 1-3.

(a) The effect of the low and high protein diets was tested on a normotensive unoperated pregnant dog (V-43). The several dietary changes had no effect on the blood pressure even when repeated with sodium chloride added to the meat.

¹ We are indebted to Dr D J Cohn of the Department of Chemistry for these determinations.

(b) The substitution of the low protein diet for the standard diet, and the later substitution of the high protein diet had no effect on the blood pressure of two dogs (Y-1, Y 80) with normal blood pressure and relative renal excretory insufficiency. As expected, the blood non protein nitrogen rose during the high protein phase of the dietary regime. (This is illustrated for Y 80 in Fig 1)

(c) In three dogs with normotension and relative renal excretory insufficiency due to partial renal arterial occlusion (W-67, W-87, U-45) six experi-

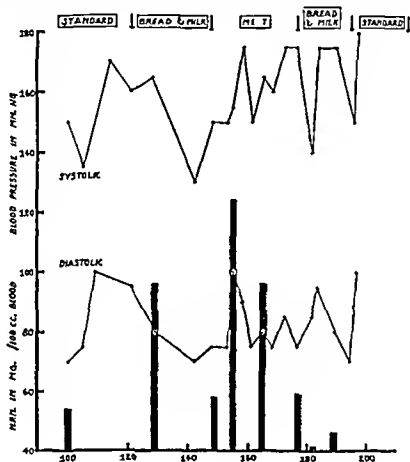


FIG 1

ments were carried out. In the first series of these, sodium chloride was added to the meat diet. In the second phase the meat was fed alone. In all instances a rise in non protein nitrogen occurred without a change in the blood pressure. In a fourth dog (W 77) an elevation in systolic and diastolic pressures was seen while on the meat regime after the animal had failed to show a rise in pressure on the meat and salt regime. The blood pressure rose from an average of 145/80 to an average of 170/100 during the high protein diet. This interesting response led us to twice repeat the dietary sequence, in neither of subsequent dietary regimes did the animal respond with an increased blood pressure, yet in each instance the blood non protein nitrogen was increased during the period of high meat intake (Fig 2)

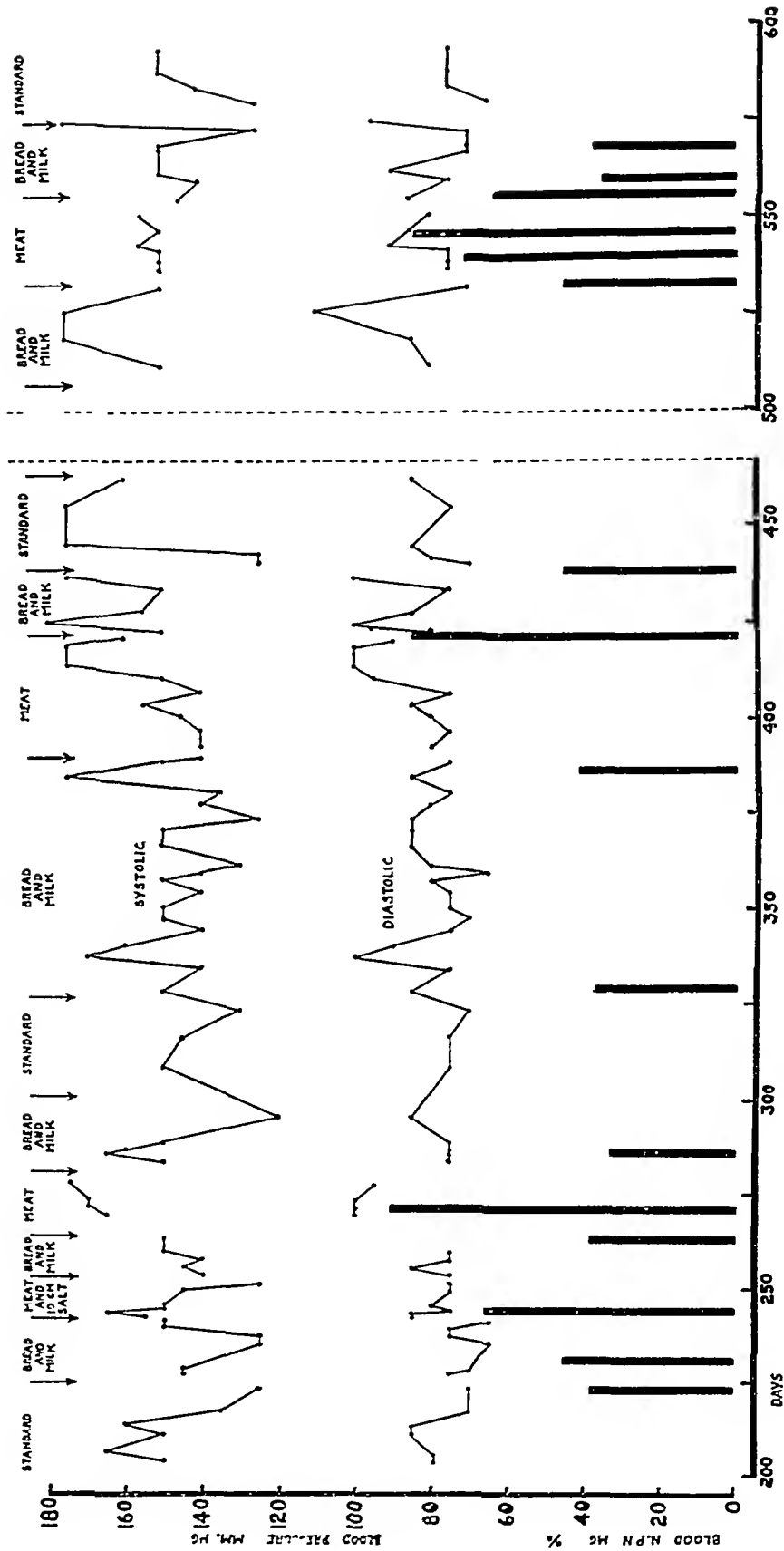


FIG 2

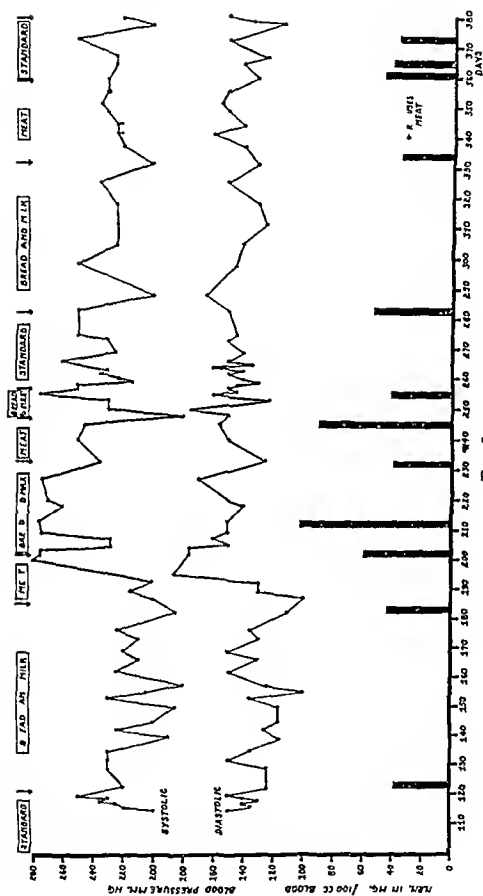


FIG 3